VACCINE PRODUCTION AS A UNIT PROCESS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE TECHNISCHE WETENSCHAPPEN AAN DE TECHNISCHE HOGESCHOOL TE DELFT, OP GEZAG VAN DE RECTOR MAGNIFICUS IR.H.R. VAN NAUTA LEMKE, HOOGLERAAR IN DE AFDELING DER ELEKTROTECHNIEK, VOOR EEN COMMISSIE UIT DE SENAAT TE VERDEDIGEN OP WOENSDAG 24 NOVEMBER 1971 TE 14 UUR

DOOR

PAULUS ALOYSIUS VAN HEMERT

SCHEIKUNDIG INGENIEUR
GEBOREN TE VOORBURG

DIT PROEFSCHRIFT IS GOEDGEKEURD DOOR DE PROMOTOR
PROF.DR.T.O. WIKÉN

DIT PROEFSCHRIFT WERD BEWERKT OP HET
RIJKS INSTITUUT VOOR DE VOLKSGEZONDHEID

-et renovabis faciem terrae-

Aan de nagedachtenis van mijn ouders

Aan mijn vrouw

Aan mijn kinderen

drukkerij elinkwijk, utrecht

CONTENTS

CHAPTER 1

Introduction

This thesis deals with the production of vaccines. More specifically, with the manufacturing of vaccines for human application. It encompasses more than a decade of study in the field. In this period the character of the Vaccine Department of the Rijks Instituut voor de Volksgezondheid in Bilthoven has changed considerably. This has been due, not only to the knowledge gained during the study of the variables of the processes involved, but especially to the better insight gained in the technological aspects of vaccine production. Because of the great financial and technical considerations, the work described here involved the cooperation with many others.

The necessary investments would never have been made if the Board of Directors of the Rijks Instituut voor de Volksgezondheid had not believed, in advance, in the applicability of the concepts concerned. *Dr. H.H.Cohen,* in his responsibility for the vaccines produced by the Institute, stimulated to a great extent the development of the concepts. A complete laboratory was at my disposal in which particular mention must be given to *Ir.A.L.van Wezel* and *Mr. P.Smid* who were collaborating almost daily in discussing the design of experiments, and also in performing them. In addition, a small staff of construction designers was present, a rather unusual asset for a laboratory; in this team the activity of *Mr.J.van Hooijdonk* cannot remain unmentioned. The detailed development of the technical outfit to be described in Chapter 3 could never have been realised without the presence of this staff.

The discussions with the promotor *Prof.Dr. T.O.Wikén,* on the preparation of the text, have been most useful, and were welcomed by the author.

Whereas a thesis is commonly the result of endogenous stimulation, this book has also received exogenous impetus insofar as colleagues in the field of

vaccine production have frequently suggested that they would welcome a comprehensive treatise of the work done in Bilthoven on this subject. For this reason, digressions, as usually encountered in a textbook, have been somewhat indulged in by the author. While covering a rather wide field, it has only appeared feasible to work out the subject to the whole depth in a certain limited number of cases. The book not only contains the record of a piece of scientific work, including a good deal of applied technical science, but also a philosophy: the concept of Unit Process.

The leading thought of this thesis is a logical consequence of the observation that processes used in the preparation of several vaccines have more features in common than is generally recognized. This led, as will be explained in Chapter 2, to the application of the concept of unit process to vaccine production.

Data on general methodology and applicable to the great majority of the specific processes leading to the production of vaccines are treated as "Basic Tools of the Unit Process" in a separate chapter (Chapter 3). It is only after this display of the "a r m a m e n t a r i u m", that a number of specific processes are detailed (Chapter 4). They serve as examples, and form the justification for the application of the principle of Unit Process.

Production of vaccines in large quantities, requiring a technological approach, becomes necessary when systematic vaccination of larger parts of a population is considered. An effective systematic vaccination programme requires a vaccine of proper effectiveness, as well as the possibility to establish a vaccination programme and to obtain a sufficient supply of the vaccine for such a programme. The programming depends upon the administrative accessibility of a population and the vaccine supply on technology. Apart from smallpox vaccination, and the programmes conducted in the armies during both world wars (typhoid and tetanus; the latter only in World War II), and diphteria immunization in the late thirties, systematic vaccinations as such were only started after World War II. Field trials, as described by *Cockburn* (1955) for pertussis in England, and by *Francis* (1955) for poliomyelitis in the United States, have contributed greatly to stimulating systematic vaccination, whereas the introduction of combined vaccines, such as the diphtheria-tetanus-pertussis-polio--combination (*Brandwijk et al.,* 1961) have simplified the application.

Statistical data concerning coverage of the population by vaccination, even within highly developed countries, are not always obtainable. An estimated value of 50 to 90 per cent for polio and smallpox in Europe and North America, and eventually the same for pertussis, diphtheria, and tetanus may be assumed. This implies that only 20 per cent of the total world population of more than 3 milliard people has been reached by the most important

vaccines, and this only during the last ten years. Smallpox vaccine forms an exception. The smallpox eradication programme of the World Health Organization has gradually brought the figure for smallpox vaccination on a considerably higher level during the last five years.

A rapid expansion of the production of vaccines must therefore be expected in the next decades, not only of the above mentioned, but also those for use in special disease areas, such as vaccines against yellow fever and those that have been developed more recently (see review by *Edsall*, 1961) such as measles and rubella vaccines. This requires that the technology of vaccine production should be considerably improved. This should take into account improvement not only in quantity but also in quality. This will be explained below in terms of consistency.

In order to give an impression of the culture volumes concerned in mass vaccine production, in Table 1-1 the culture volume for the preparation of one total human dose is given for each of several vaccines. The data are approximations, depending not only on the yield of cells or product per unit volume, but also on the dose administered; if several, strongly different, doses of one vaccine are present, as for instance when oral vaccination of

Table 1-1 Approximate culture volumes (in ml), required for the preparation of one total human dose (t.h.d.) of several bacterial and viral vaccines. For explication, see text.

type of vaccine	inoculations per t.h.d.	ml cult. per t.h.d.
pertussis	3	1.0
diphtheria	3	0.3
tetanus	3	0.3
staphylococcus α	2	0.5
cholera, parenteral	2	0.2
cholera, oral	2	2
typhoid, parenteral	2	0.02
typhoid, oral	2	2
B.C.G.	1	0.02-0.1
polio, inactivated	3	6
polio, live	3	0.1
smallpox	1	0.03
measles, inactivated	3	1.0
measles, live	1	0.003
rubella, live	1	0.1

enterobacterial vaccines is compared with the parenteral route, two data are given. For viral vaccines the most modern method of preparation is chosen; so the smallpox figure is derived from tissue culture, as the classical method on calf's skin cannot be evaluated in terms of volume. Furthermore, for some virus vaccines the figure is based on a method in homogeneous culture, which is under development (see Chapter 4.3). The table shows that, with the exception of live virus vaccines, considerable volumes are required. Larger volumes than the ones mentioned here will be needed, if purification procedures, such as envisaged for instance for pertussis vaccine, would be practised.

Directing our attention to the technology of vaccine preparation, we must state that in the early years vaccines were being prepared in a laboratory on a laboratory scale and with laboratory methods. This picture seems to persist more than is desirable because of some features inherent to vaccine production:

1. Much of the early development has been done by government. Preparation took place in national institutes or other non-profit organizations. The preparation was done on the bench of the medical bacteriologist; efficiency and productivity were only of secondary concern.
2. Whereas, for instance in the production of antibiotics, the culture volumes involved are so large that efficient technological procedures have been necessary almost from the beginning, in vaccine production a somewhat intensified laboratory procedure could meet the demand, even for a population of, say, 50 million people.
3. Virus vaccines, prepared routinely on such substrates as calf's skin and chicken eggs, lend themselves poorly to industrial preparation. Even the use of monolayer cultures of trypsinized tissue cells does not provide a solution in this respect. Only lately, with the introduction of homogeneous tissue culture techniques, a possible break-through has been made.
4. The complex cultural conditions of many pathogenic microorganisms and viruses has frequently led to the belief that cultivation in "tanks" was either impossible or extremely difficult to perform.

The characteristic of laboratory cultivation of microorganisms is, in this context, *growth on a small amount of substrate, at a constant temperature.* Variables such as pH and real incubation time (that is, the time to reach a defined physico-chemical and biological condition, cf. p.) are seldom controlled. This method of cultivation is not ideal for producing vaccines of good consistency (for a definition of consistency, see p. 17). The reason is

that the reproducibility of the process cannot be checked adequately due to lack of criteria.

Consistency is becoming more and more a necessity for the following reasons:

1. Extensive field trials such as those previously mentioned can give dependable results only if the vaccine used is of constant quality. One may expect similar results in further vaccination programmes only if later supplies of vaccine are of the same quality.
2. More efficient tests of potency and safety, such as the mouse protection test for pertussis vaccine and the tremendous safety control tests for virus vaccines such as polio, enable us to distinguish between good and bad vaccines.

The characteristic of industrial cultivation of microorganisms, on the contrary, is that an efficient way of scaling-up is present and that enough variables can be measured to ensure the exact reproduction of the process. Only in this way may a consistent product be expected. Both these requirements can only be fulfilled by cultivation in a homogeneous system, as will be explained in the introduction of Chapter 3.

CHAPTER 2

Group Characteristics of Vaccine Production

Turgueniew stated in 1857 that "systems attract those who do not succeed in capturing truth in its totality...". I am, however, of the opinion that it is in many instances advantageous to bring all aspects of a subject into a system. A system may help to find the features that are in common in a group of phenomena. It may simplify finding a common approach in cases where this did not seem clear before. A system should only serve, however, to guide thoughts; it may never become autonomous and imperative obliterating n u a n c e s that may be important.

In the early time of chemical technology, *Lewis* (1923) tried to create order in the multitude of activities leading to a finished product, by the designation Unit Process and Unit Operation. Examples of the first are chemical reactions such as oxidation, hydroxylation and alkylation; the second is exemplified by operations like filtration, distillation and centrifugation. This system had the advantage that both entities could be described independently of the particular procedure, thus establishing knowledge of more general application. Working out a specific procedure is thus brought back to the choice of materials and conditions; with other words to making variations on a known theme.

Although the procedures used for the preparation of vaccines are of quite another nature than the chemical processes on which *Lewis* applied his system, we felt that the term "Unit Process" was applicable m u t a t i s m u t a n d i s to vaccine production.

For the application of the concept of "Unit Process" it is required that the procedures concerned have some group characteristics in common. In the case of vaccine production these are:

1. A cultivation of bacterial or tissue cells, at temperatures around 37°C in media that are sometimes considerably complex.
2. A cultivation volume that is small, when compared with other industrial cultivation procedures, volumes being in the order of 25-1000 litre.
3. The need for strict asepsis, as well as protection of the operator against infection with the microbe under cultivation. The first consideration is made because most organisms involved are cultivated in media in which they are easily overgrown by common contaminating bacteria; the second because the laboratory strains used are often considered still to be pathogenic to man.
4. The production of a substance that is not a main metabolic product such as ethanol from yeast or citric acid from **Aspergillus niger**. This is true whether the end product consists of cells or is a bacterial product separated from the cells. In both cases it is the formation of one or more antigens, of a complex chemical nature, that counts.
5. The obtainment of an end product with a maximum of safety for man, which is an important feature. Vaccines belong to the preventive medicaments. Especially when the disease, against which one is immunized has almost completely disappeared, the side-effects of the immunizing agent, applied to healthy persons, come into focus.
6. Because it is in most cases impossible to determine the potency of the vaccine during the cultivation process, more than usual emphasis is laid upon reproducibility of the process and hence on determination of process variables.

In studying the literature on the procedures published for preparation of the different vaccines one cannot help to be struck by the fact that each vaccine is considered irrespective of the data on other vaccines that could be applicable. The best illustration is the fact that apparatus have been described for the cultivation of for instance **Corynebacterium diphtheriae** or **Brucella abortus**, that have no detail specific for the microorganism mentioned. Hence, up to the present in several institutes, the production of different vaccines is performed in separate rooms by different personnel using different methods without any justification for this splitting in the closely related basic procedure.

The application of the concept of Unit Process requires a general consideration of all materials and methods that are common to vaccines, leaving only those details that are specific for the preparation of a certain vaccine to special treatment.

The concept is visualized in Fig. 2.1. The feature most obviously belonging to the Unit Process is the cultivation apparatus, including the instrumentation for the measurement and control of physico-chemical

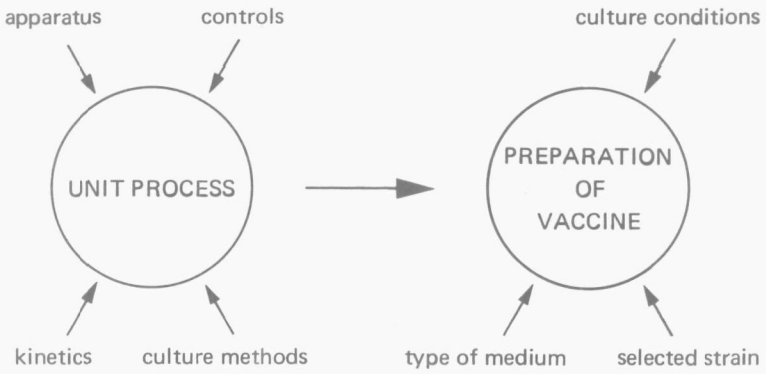


Fig. 2.1 The concept of Vaccine Production as a Unit Process.

variables. We have shown that one set of apparatus will give an optimal solution for the cultivation process of all bacteria (and tissue cells !) concerned. In addition, a number of aspects of the culture methodology is shared by all organisms studied. This also applies to the especially difficult position in regard to the kinetic approach of the production of such complex compounds as antigens.

Thus the aspects left for individual treatment are the selection of strain and medium, and the choice of the optimal culture conditions for the production of a certain vaccine.

CHAPTER 3

Basic Tools of the Unit Process

In the foregoing chapter the choice of the "Unit Process" principle as an approach to the production of vaccines has been reasoned. It has served to guide our thoughts. The material basis, however, which was necessary for the practical study of the cultivation processes involved, still had to be provided.

A cultivation process presupposes the application of appropriate techniques, and these techniques can only be studied if suitable equipment is available. This applies not only to the container in which the microorganism is allowed to multiply and form the desired product, but also to the auxiliary instruments that enable us to measure, indicate, register and control physico-chemical variables. The importance of these variables in the culture processes studied cannot be overestimated. They give continuous information with almost no time delay. Knowledge of these parameters provides the basis for selecting optimal circumstances, thus ensuring repetition of the optimal values in subsequent cultivations. It is on this last phenomenon that the consistency of the product, i.e. the vaccine, depends. Consistency in this context means the repeated formation of an end product of good quality without occasional inferior batches. One has to depend upon exact repetition of the procedure to ensure consistency, because some vaccines are difficult to test for efficacy and innocuity, as will be stressed in the next chapter. The control of physico-chemical variables is therefore an indispensable requirement.

There are two reasons for considering homogeneous culture as the only acceptable way of cultivation. The first directly bears upon the indispensability of physico-chemical variables in this Unit Process. The value measured only gives information about the spot where the probe is inserted; if the

physical conditions in this location can be considered representative for the whole culture, valid conclusions can be drawn about the process. This is only the case in a homogeneous culture i.e. in a culture in which all constituents are uniformly distributed. In microbiology the expression "microenvironment" is used to designate the part of the universe that is "seen", in a physico-chemical sense, by a microorganism i.e. the part in which a direct interaction between microbe and surrounding exists: only if all microenvironments of a culture are identical it can be considered as homogeneous.

This homogeneity is not going down to the molecular level. The microorganisms themselves, being particles of the order of magnitude of 1 μ are present. Sometimes small clumps of bacteria are almost impossible to avoid, as with **Mycobacterium tuberculosis** (see p.113). In the cultivation of tissue cells, the cells are in the order of 10 μ. When cultivated on the so-called "microcarriers" (see p.156) clumps of 100-200 μ in diameter are regular. With effective mixing with mechanical stirrers there will be an even distribution of particles, and the use of the term homogeneous is justified. Likewise there is no complete homogeneity attainable of the concentration of those compounds that are diffusing into and out the cell in connection with its metabolism. A certain concentration gradient will always occur. If the gradients around all cells are of the same character, due to sufficient mixing, the term homogeneous is again applicable. The same holds true for the undissolved gases present as bubbles in the culture. An even distribution, and identical gradients around each bubble give the culture a homogeneous character in the sense of this treatise. It is understood that it is not easy to define how a culture should be agitated in order to achieve satisfactory homogeneity. One is referred to the work of *Hansford* and *Humphrey* (1966).

The second reason to choose a homogeneous culture is as following: assuming that there is an optimal condition for a microorganism to fulfill a certain task of reproduction or production, then all members of a bacterial population should be kept in this condition. And this, again, can only be achieved in a homogeneous culture. The inadequacy, in this respect, of a culture on a solid medium is so obvious (compare the condition of a cell in the top of a colony with one in the middle !) that it is difficult to understand that the use of solid culture is still firmly defended in several cases in vaccine production. For pertussis vaccine production, the opposing views appeared clearly during a lively discussion on the formulation of conclusions and recommendations for pertussis vaccine preparation at the Pertussis Conference Bilthoven (1969; see Symp.Ser.immunobiol.Standard. **13** (1970) p.273).

For the fulfillment of our purpose it was considered necessary to set up a system, all components of which were tuned to each other. In the early

sixties when this work started no commercially available apparatus was considered satisfactory. Therefore we decided to design the equipment ourselves. In the course of ten years much work has been done, illustrated by the fact that more than two thousand drawings have been made by the staff of the Rijks Instituut voor de Volksgezondheid carrying out this project. The system not only comprised a set of cultivation vessels of increasing capacity but also other vessels for auxiliary purposes, and all the necessary instrumentation, brought together in panels.

A few basic principles were maintained throughout the development:

1. The use where possible, of commercially available components, provided they are well standardized and of satisfactory constant quality. This applies to simple parts such as O-rings and glass containers, as well as to complicated components such as electronic measuring and controlling circuits.
2. Interchangeability of parts within a group of apparatus, and also with comparable parts in other groups. Insofar as it involved newly developed parts it implied a rigid system of production control, using accurate measuring devices and calibrating reference parts. Examples are the inlets for stainless steel tubes, and the lids for the standard glass vessels.
3. Arrangement in the final system of all components in a compact and surveyable way.

In this chapter the cultivation vessels will be dealt with first, together with some containers that have directly been derived from them. In a separate section the measuring principles and the different combination panels will be described.

Theory and practice of cultivation techniques (batch and continuous) conclude the chapter.

3.1 Cultivation Vessels

The first impetus for the development of fermentors of laboratory and pilot plant scale was given, in the years after 1945, by the antibiotics fermentation industry. Designs for more specialized purposes eventually followed.

A critical summing up of conditions for a good fermentor is given by *Malmgren* and *Hedén* (1952a). A survey of the most important literature up to 1957 has been written by *Fuld* and *Dunn* (1958), whereas a great deal of information about apparatus for continuous culture has been collected by *Řičica* (1958, and also later reviews of *Málek* and *Řičica*, e.g. 1970 a and 1970 b). In addition to the literature quoted in the following, one is referred to the articles of *Brown* and *Peterson* (1950b), *Callow* and *Pirt* (1961), *Chain* et *al.* (1952; 1954), *Dispigno* (1961), *Feustel* and *Humfeld* (1946), *Fortune* et *al.* (1950), *Hedén* (1957; 1962), *Hedén* and *Malmborg* (1958), *Hedén* and *Holme* (1961), and *Slezák* and *Sikyta* (1961). Critical reviews of commercially available laboratory fermentors have been made by *Solomons* (1967; 1971).

The most common design in these early days was a cylindrical glass tube or vessel, enclosed between two stainless steel plates held together with two long screws; see *Bartholomew* et *al.* (1950), *Brown* and *Peterson* (1950a), *Rivett* et *al.* (1950), *Humfeld* (1947), and *Málek* (1961). A variation on this concept is the type of fermentor described by *Elsworth* et *al.* (1956; 1958).

It consists of glass pipeline fitted with stainless steel plates at top and bottom. Free hanging glass vessels fitting against a top lid with a similar flange construction are described by *Lumb* and *Fawcett* (1951) and by *Řičica* (1958). Our own fermentor, to be described in the following, is of this type. It most closely resembles the small stainless steel fermentors, as described by *Friedland* et *al.* (1956), *Nelson* et *al.* (1956), *Falini* (1960), *Malmgren* and *Hedén* (1952 b), *Fuld* and *Dunn* (1958), and *Joó* et *al.* (1959), and shares with these ease of assembly and handling, but has the advantage of free visibility of the process through the completely accessible glass walls. *Elsworth* objects to the use of a glass bottom on the grounds that the overflow cannot be brought through it. We have overcome this by using an overflow consisting of an U-bent tube introduced through the top.

The earliest fermentors mostly had packed stuffing boxes (see *Elsworth* et *al.*, 1956) as stirrer shaft seals. Better solutions to this problem are:

1. The radial mechanical seal, as described, for instance, by *Elsworth* et *al.*

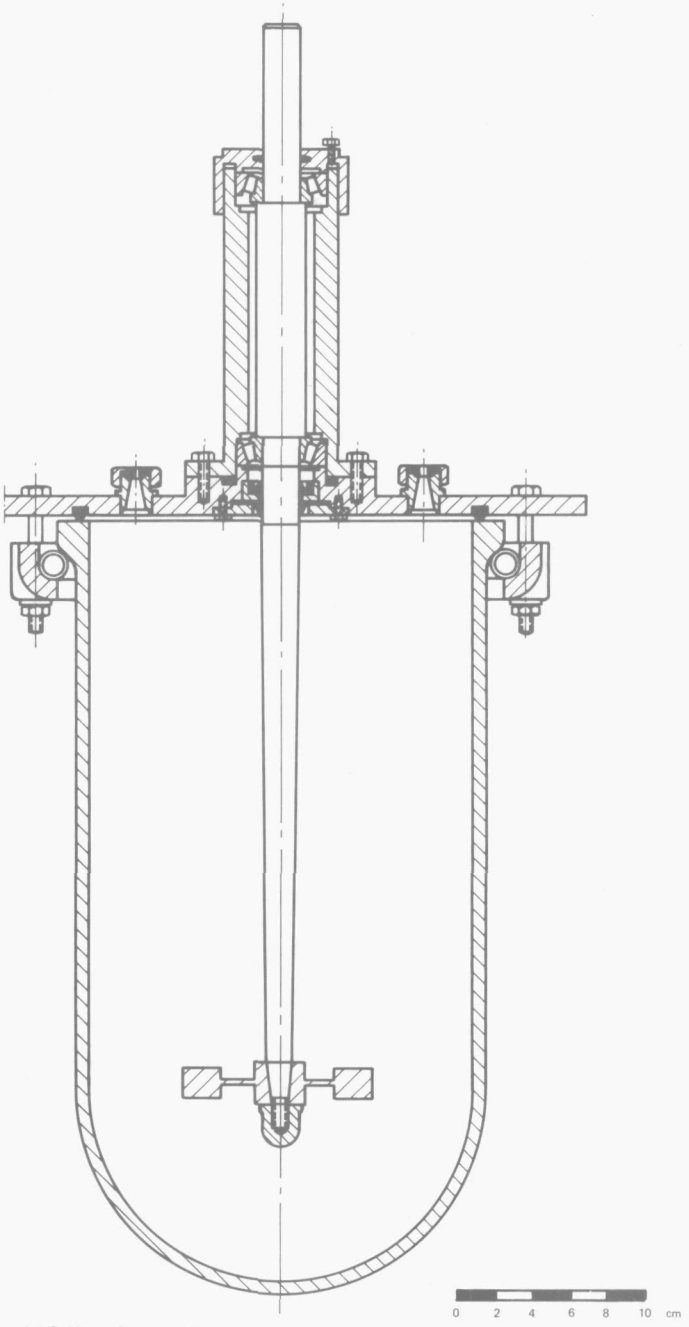


Fig. 3.01 Cross section of 10 litre fermentor.

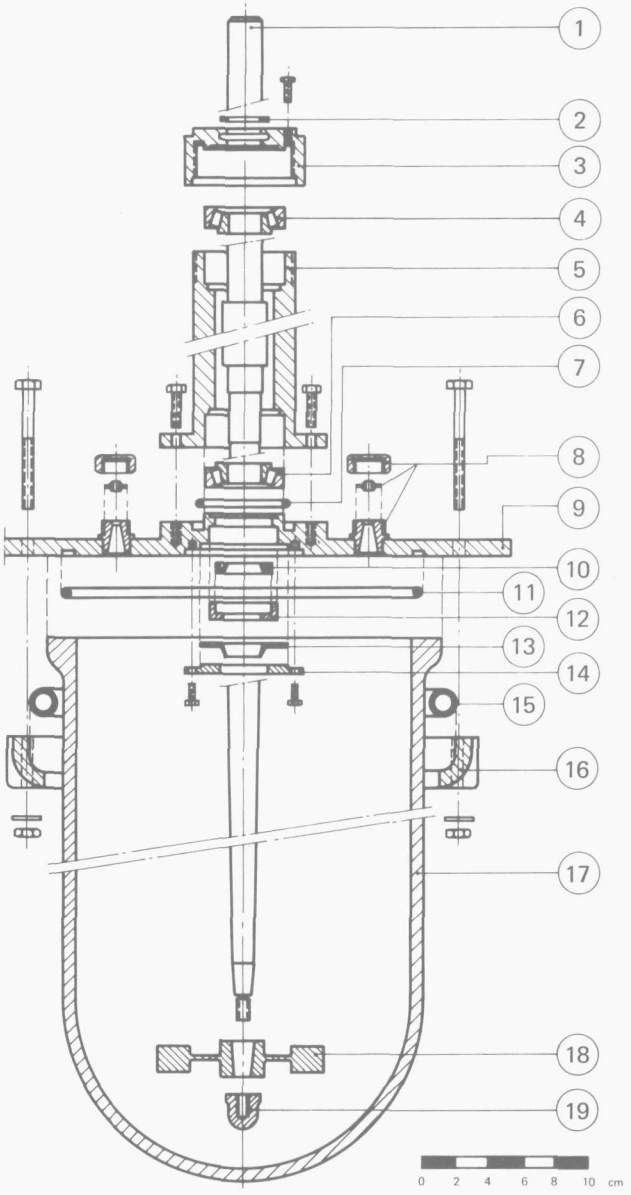


Fig. 3.02 Exploded view of 10 litre fermentor.
1. stirrer shaft; 2. felt oil-seal; 3. screw-cap for adjustment of roller bearings; 4. roller bearing; 5. housing for roller bearings; 6. roller bearing; 7. O-ring; 8. inlet for stainless steel tube; 9. stainless steel lid; 10. oil-seal (Simmerring); 11. O-ring; 12. housing for Simmerring; 13. additional oil-seal (Hutmanschette); 14. ring for positioning Hutmanschette; 15. rubber lining; 16. flange, light metal; 17. glass vessel; 18. interchangeable impeller; 19. cap nut.

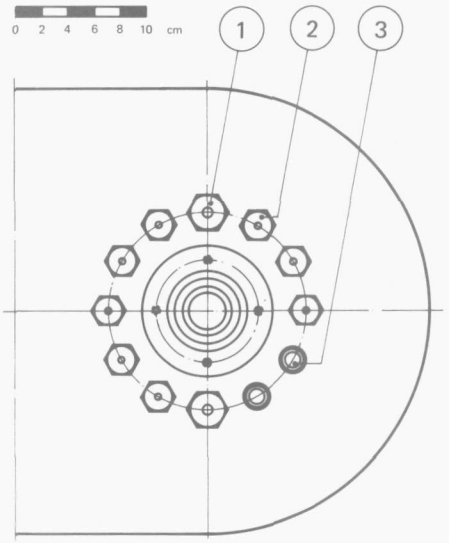


Fig. 3.03 Lid of fermentor, seen from above.
1. Inlet for 8 mm o.d. tube.
2. Inlet for 6 mm o.d. tube.
3. Inlet for pH- or pO_2-electrode.

(1958) and also used by *Friedland* et *al.* (1956), *Malmgren* and *Hedén* (1952 b), and *Hedén* (1958).

2. The oil seal of the type used frequently in motorcars, as applied by *Kroll* et *al.* (1956), *Lumb* and *Fawcett* (1951), and most probably also by *Málek* (1961).
3. Instead of seals, a magnetic coupling is suggested recently and already applied to some commercial types of small fermentors.

As the most simple and effective solution to the problem of shaft sealing, we have adapted the oil seal, improving the standard application.

Other features of small fermentors which have been subject to gradual development are heating and sterilization methods. Almost always the earlier fermentors were kept in a waterbath, and several commercially available fermentors are still of this type. The disadvantages are the risk of temperature differences between waterbath and culture fluid, decreased visibility of the vessel contents, and an extra contamination hazard.

Stainless steel fermentors, even relatively small ones of 100 litres can easily be equipped with a jacket, both for sterilization and for thermostatic regulation. For the small glass vessels, good solutions have been found by *Elsworth* et *al.* (1956), *Řičica* (1958), *Málek* (1961), and *Fiechter* (1962). The heating unit consists of water coils or electrical heating elements, or a combination of both.

Most investigators, however, sterilize their small glass fermentors in the

autoclave, accepting thereby the complications of reattachment of driving unit and connections after sterilization. *Elsworth* et *al.* (1958) sterilize their improved fermentor by blowing live steam through the interior, filling in the medium aseptically afterwards. As far as we are aware, no author has described a small glass fermentor to be sterilized in situ with the medium. It is the solution we have chosen for our fermentor.

Most fermentors, glass as well as stainless steel ones, have relatively primitive tube connections. In some cases they are simply welded in the lid, making them completely inexchangeable. In other cases they can be fastened by some type of union joint making them exchangeable, although it is still difficult to adjust them in every possible position. Improvements have been made by *Elsworth* et *al.* (1956), using O-rings, and by *Hedén* (1958), who developed special "Stericonnectors". The latter devices, perfectly designed as they are, are complicated and difficult to apply to small vessels with limited lid dimensions. The argument of complicatedness applies a f o r t i o r i for the inlets designed by *Fiechter* (1962) and now adapted by two Swiss manufacturers. The design of our tube connections is described on p. 27. It has been used on all equipment, and is, without any adaptation, able to fit manometers, safety valves, etc.

The advantages of glass vessels are obvious: the contents can be inspected, which is of paramount importance in developmental work.

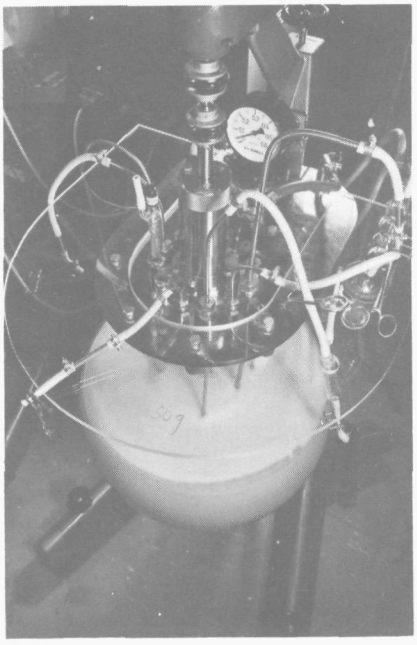


Fig. 3.04. 50 litre fermentor with a culture of **B.pertussis**

The following considerations apply to the volume to be chosen:

1. *The minimal size* of the culture vessel is determined by considerations of experimental technique. Not only is it difficult to place piping and electrodes in a small volume, but there will be also restrictions to size and number of samples. Biological tests frequently require considerable sample volumes, and no microdeterminations exist for them. A minimum of 2-3 litres was considered practical.
2. *The maximal size* is determined by ease of handling and by the amount of culture fiuid needed for a particular programme. Since the yearly production of most vaccines in a country with a population up to 50 million people, is in the order of thousands of litres, batch cultures of 25-50 litre and continuous cultures with volumes of 3-8 litre are the maximum needed and a set of glass vessels ranging from 1 to 50 litre has been found most practical.

In respect to size and material, tetanus toxin production forms an exception, and in section 4.2.2 stainless steel vessels of a size of 200-400 litre will be described.

In Fig. 3.01 and 3.02 a cross section and an exploded view of the fermentor developed by us (*Van Hemert,* 1964b) has been given and in Fig. 3.03 the disposition on the lid is shown. Fig. 3.04 gives an impression of a practical set up with the 50 litre vessel. Fig. 3.25 gives a picture of the 10 litre fermentor.

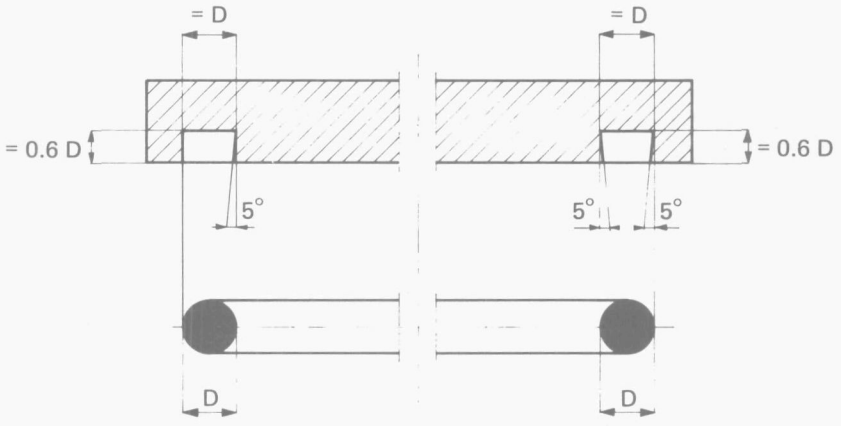


Fig. 3.05 Recommended dimensions for O-ring grooves in glass/metal joints. Left: flange joint. Right: screw-cap joint. D = diameter of O-ring.

The fermentor consists of a stainless steel lid, in which are located the stirrer shaft with seals and roller bearings, the pH-electrodes and the pipes for transport of fluids and gases and for temperature control. To this lid, the glass vessel is attached by an O-ring seal.

The glass part is composed of industrial Jena glass with a flat flange of the so-called KF type. Sizes of 5, 10 and 50 litre are available with the same flange size of 200 mm.

An hermetical seal between flange and glass is achieved by an O-ring placed in a groove in such a way that, even after compression that causes the O-ring to fill the groove, no contact between metal and glass is caused. For this purpose the relative dimensions as shown in Fig. 3.05 have been found optimal.

The stirrer consists of a stirrer shaft rotating on two conical roller bearings enclosed in a cylindrical housing screwed on top of the lid. Aseptic sealing is accomplished by a shaft seal (Simmerring) and a hatshaped seal (Hutmanschette). Both are easily replaced, but at least 3000 hours operation, including several sterilizations, can be effected without renewal. As both seals exert a high surface pressure on the shaft, hardening of the part in contact with the seals is needed to avoid rapid wear. The stainless steel types 304 or 316 that are in frequent use where high corrosion resistance is

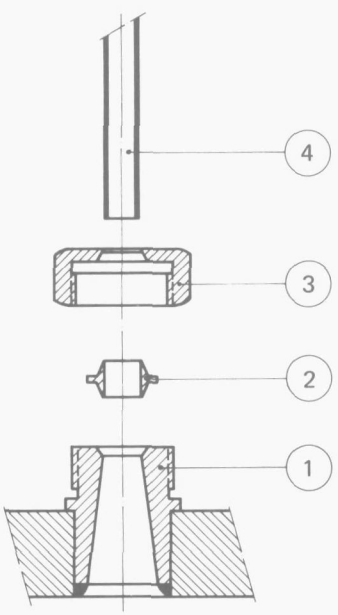


Fig. 3.06 Standard inlet for stainless steel tube (shown is 8 mm tube; inlet for 6 mm tube is similar).
1. Stainless steel sleeve, welded in stainless steel lid. 2. Saturnus ring (nylon). 3. Gland nut (brass). 4. Tube.

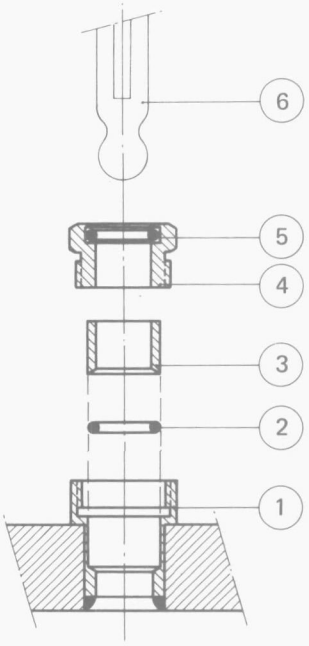


Fig. 3.07 Standard inlet for pH- or pO_2-electrode.
1. Stainless steel sleeve, welded in stainless steel lid. 2. O-ring (perbunan). 3. Gland (stainless steel). 4. Gland nut (brass). 5. O-ring (perbunan). 6. Electrode.

required, cannot be hardened. A special type (e.g. DIN 1.4122) is therefore used. Alternatively the shaft is either made of this material, or it is made of two parts, pressed into each other, the lower part being of 304 or 316, which have better corrosion resistance. The impeller is placed on the calibrated conical end of the shaft and held in place by a cap nut.

Because in- and outlets to fermentors have to be changed frequently to adapt them to a particular process, an arrangement guaranteeing maximum versatility was considered most suitable. Fig. 3.06 gives a schematic representation of such an inlet. Every commercially available stainless steel tube, provided its outer surface is smooth, and has a diameter tolerance of ± 0.1 mm, is suitable. Tubing can easily be bent in a vice and cut to the desired size. The nylon washer ("saturnus ring") has proven, after careful experimentation, to be the simplest solution for a hermetic seal; it can be used throughout many cycles including steam sterilization.

For the placement of electrodes e.g. for pH and for dissolved oxygen, another special inlet has been designed, as shown in Fig. 3.07. Here two O-rings are used to guide the glass stem of the combined pH-electrode without touching metal parts.

Besides the stirrer shaft and the two electrodes, ten pipes can be inserted,

of which eight are 6 mm o.d. pipe and two are 8 mm o.d. pipe. In a typical process they are used as follows:

1. inlet heating coil,
2. outlet heating coil,
3. inlet air,
4. outlet air,
5. thermocouple,
6. inoculation,
7. sampling,
8. inlet medium, } continuous culture
9. outlet culture fluid, }
10. spare.

The fermentor lid is attached to a movable support. An electric motor with a continuously variable speed drive is placed on top of the stirrer shaft and attached independently to the same support, as shown in Fig. 3.08. From this unit the following connections, with standard fittings, lead to the instrument panel:

1. tube to heating coil,
2. tube from heating coil,
3. thermocouple cable,
4. motor cable,
5. pH-electrode cable,
6. oxygen electrode cable,
7. gas inlet tube,
8. gas outlet tube.

All cables and tubes are flexible and easily detachable: a new fermentor may be attached to the same panel connection within a few minutes.

The coil in the fermentor is used for sterilization as well as for thermostatic regulation. In the first place steam (of about 2.5 kgf/cm^2) is passed through the coil, heating the water or the culture medium in the vessel to boiling at a temperature of 110°C (0.5 kgf/cm^2 overpressure). By letting the steam out of all connections on the fermentor, one by one, they are all sterilized. The filter in the ingoing air line is steamed as the last one; immediately thereafter air (or another gas mixture) is allowed through the filter into the vessel. Regulation of gas flow rate and gas composition will be described in section 3.2.3.

For thermostatic regulation water is passed through the coil. The principle of the control itself will be described in section 3.2.1.

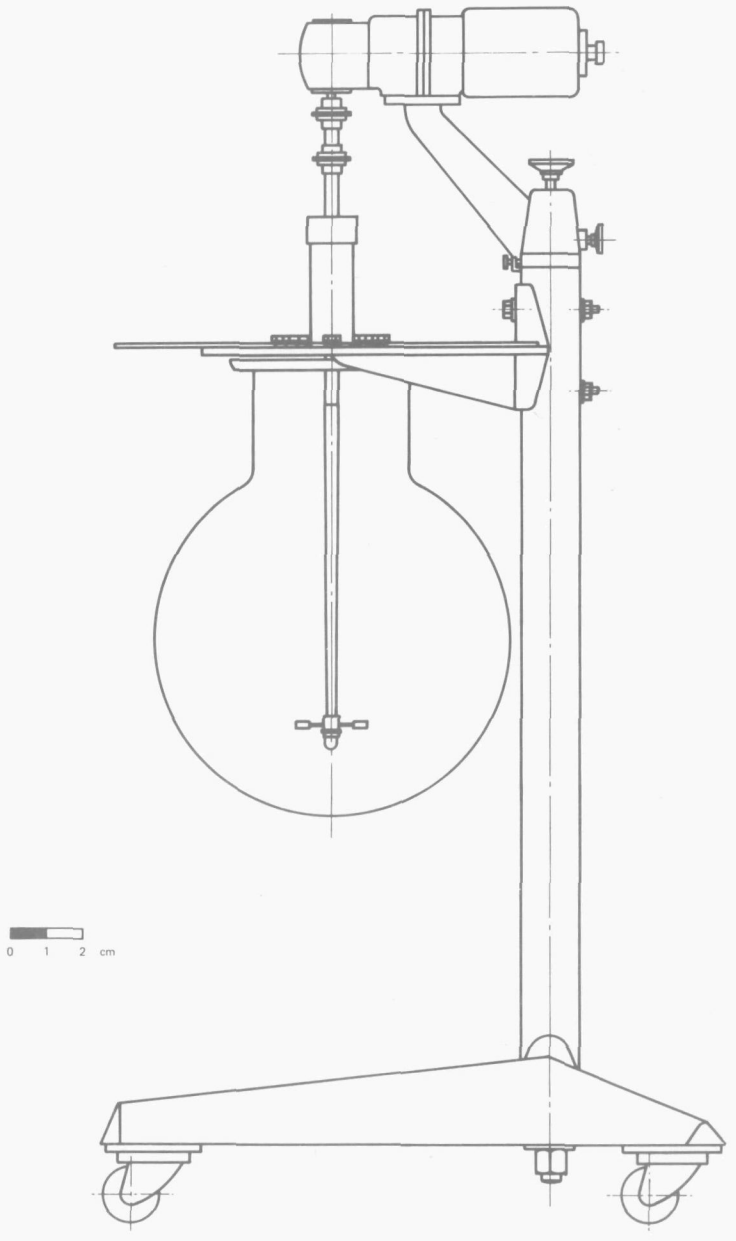


Fig. 3.08 50 litre fermentor with motor and support. Vertical cross section.

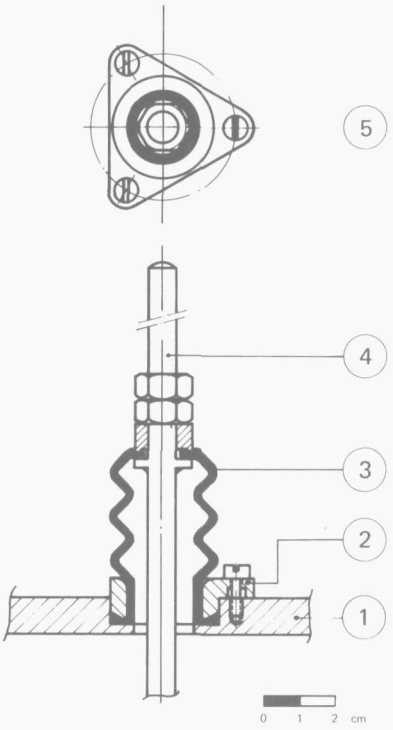


Fig. 3.09 Improved aseptic seal for small Vibromixer shafts, as used with B-containers. Vertical cross section.
1. Lid of B-container. 2. Gland with three screws. 3. Bellows (perbunan). 4. Mixershaft. 5. Horizontal cross section.

From the fermentor several other containers have been developed for purposes of vaccine production. They fall into three groups and will be described subsequently:

SO-CALLED B-CONTAINERS

They consist of a standardized Jena flask of 10 or 20 litre volume, provided with the same flat KF flange as the fermentor vessel, but of only 80 mm diameter. The lid has six standard inlets for 6 mm stainless steel tube, one of which may be replaced by an electrode inlet. The lid can be provided with a central opening for a Vibromixer shaft. Because the design of the original stuffing box was thought to be unsatisfactory in preventing contamination, a new design had to be made, as shown in Fig. 3.09. In this, the vibrator shaft is provided with a ring welded to the shaft. A bellows of perbunan is pressed against this ring with another ring. In the same way the other end of the bellows is pressed between a depression in the stainless steel lid and a ring screwed onto it with three screws. A general plan of the containers is given in Fig. 3.10 and 3.11.

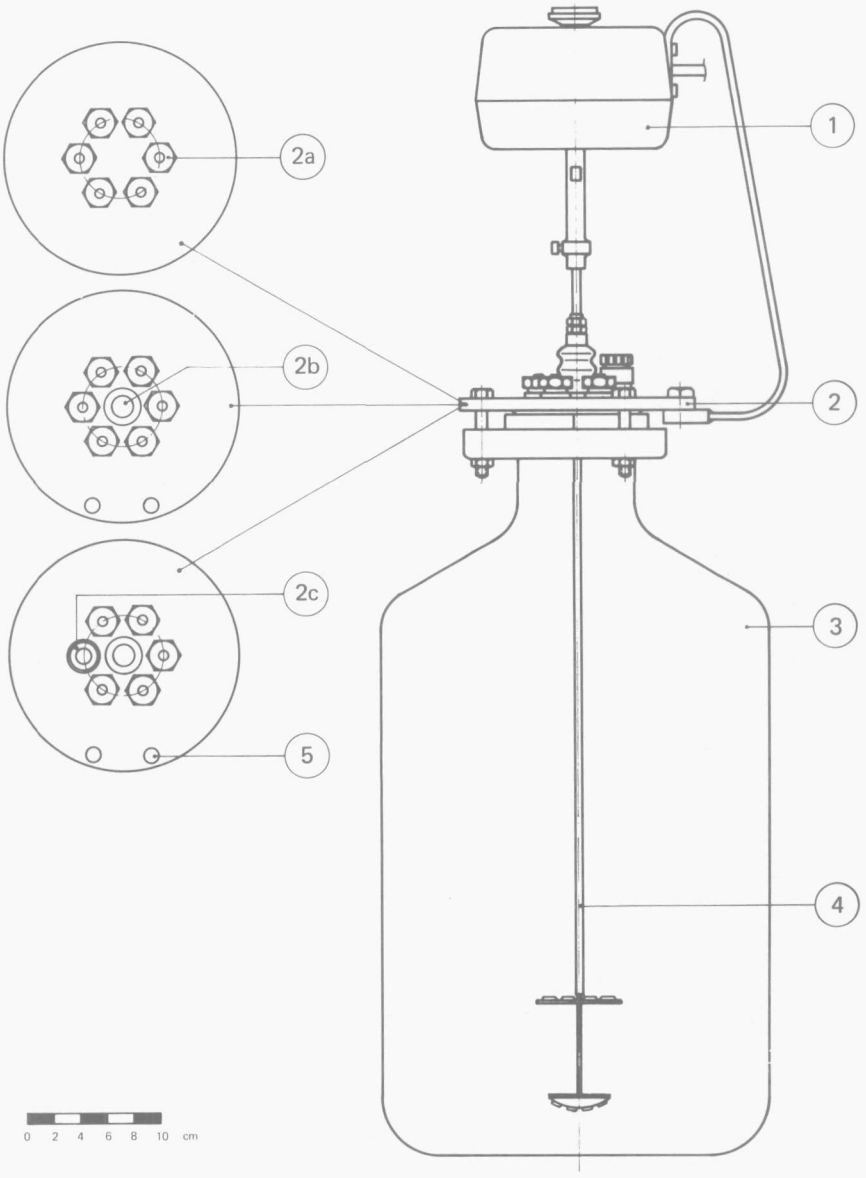


Fig. 3.10 B-container (20 litre) with Vibromixer. Vertical cross section.
1. Vibromixer; 2. lid; 3. glass flask with standardized neck; 4. mixershaft with two impeller blades; 5. points of attachment of Vibromixer.
The three lid types are shown, as seen from above:
2a. with six inlets for 6 mm o.d. tube (type H-1),
2b. the same as a, but with central opening for mixershaft (type H-3),
2c. the same as b, but one inlet changed into electrode-inlet (type H-12).

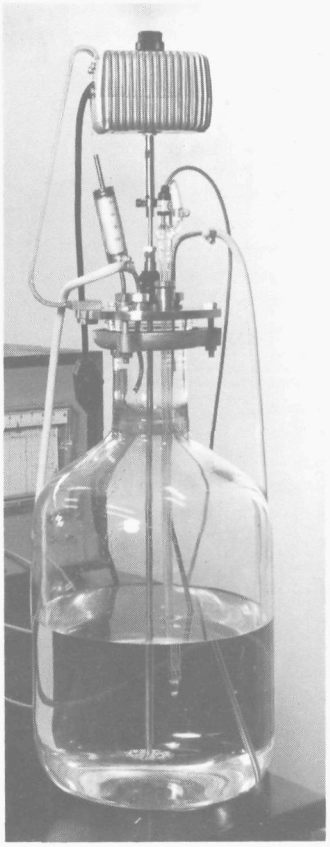


Fig. 3.11. B-20 bottle with Vibromixer

B-containers with stirrers are in general use now in our Institute (and several others) for storage of particulate vaccines, and, without stirrer, for storage of sterile fluids such as culture medium. Provided with a heating coil, thermocouple and stirrer they are used for accurate heat treatments as in detoxification and killing of vaccines. Small scale cultivations are also sometimes performed in these vessels, namely for research on tetanus toxin production. Finally, small scale vaccine mixing procedures have been performed in these containers, as for the development of quintuple vaccine (diphtheria-tetanus-pertussis-polio-measles). For this purpose a combined pH-electrode was mounted, together with a stirrer.

SO-CALLED C-CONTAINERS OR TRANSPORT VESSELS

They consist of a cylindrical Jena glass vessel of 400 mm diameter with a

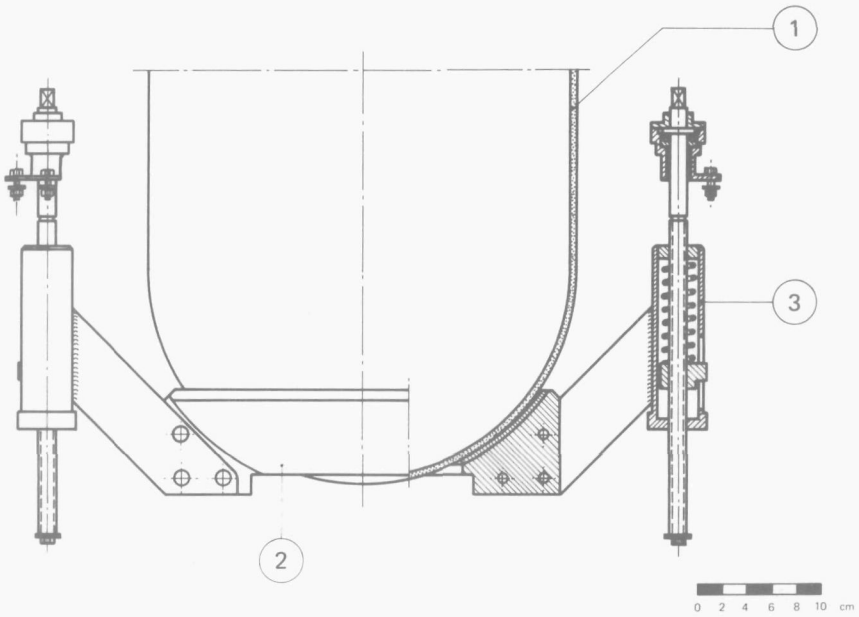


Fig. 3.12 Supporting device for C-container. Vertical cross section.
1. Glass vessel. 2. Supporting cup. 3. Spring-loaded supports (4 pieces).

300 mm flat KF flange, attached to a square stainless steel lid, hung in a movable support. Because of the heavy load the vessel is supported by a special disc suspended on four loaded spiral springs as shown in Fig. 3.12.

Again the lid is provided with standardized inlets. It can also be fitted with a Vibromixer.

Sterilization is achieved by introducing live steam into the vessel through an asbestos pad filter, placed on the lid; the same filter functions later as an air filter to equalize overpressure or to apply pressure for removing the contents. The maximal allowable overpressure of 0.55 kgf/cm^2 is controlled by a valve, which also had to be developed because no suitable type existed for this purpose. All internal parts are of stainless steel, and asepsis is ensured by an O-ring construction: cf. Fig. 3.13.

The C-containers are currently used for storage of mixed vaccine lots and for detoxification and storage of tetanus and diphtheria toxin/toxoid. They are even used in some other institutes for mixing vaccines (Statens Seruminstitut, Copenhagen; *Kn.Nielsen* pers.comm.). Fig. 3.14 gives a general impression of a C-container with Vibromixer.

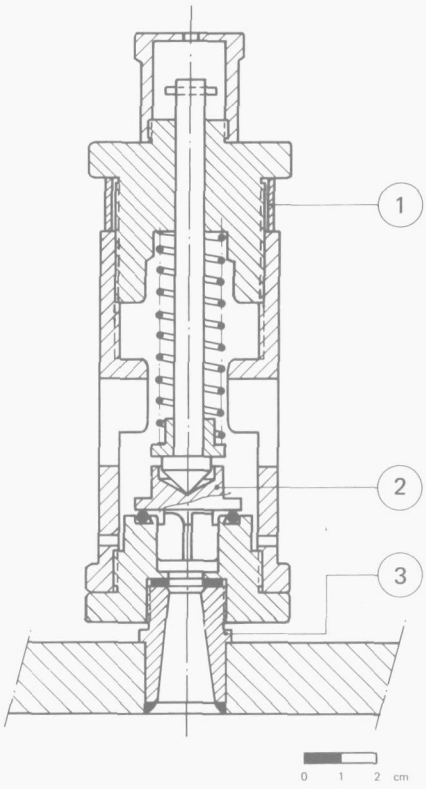


Fig. 3.13 Safety valve for aseptic work.
1. Ring for adjustment of safety pressure. 2. Stainless steel valve on O-ring seat. 3. Standard inlet for 8 mm tube.

B- as well as C-containers have been used for medium storage and also as a receptacle in continuous culture.

SO-CALLED D-CONTAINERS (MIXING VESSELS)

For the special purpose of vaccine mixing a range of three double walled stainless steel vessels, with capacities of 100, 200 and 400 litre have been designed.

They all have the same lid, the design of which is shown in Fig. 3.15. Again standardized inlets for 8 mm tube and for a pH-electrode were fitted, together with a Vibromixer shaft seal. In this case sterilization is achieved by letting steam into the jacket.

Fig. 3.15 gives a schematic representation of the 200 litre vessel and Fig. 3.16 the arrangement in the mixing room. The vessels are all placed on electronic weighing elements facilitating the mixing procedure.

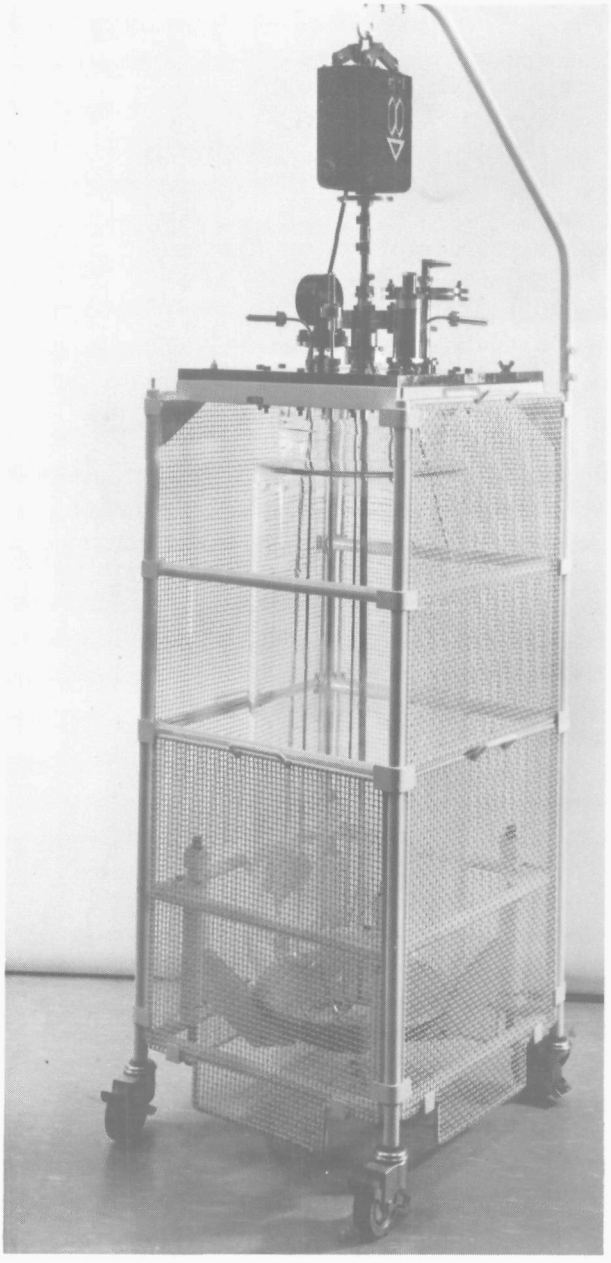


Fig. 3.14. C-container

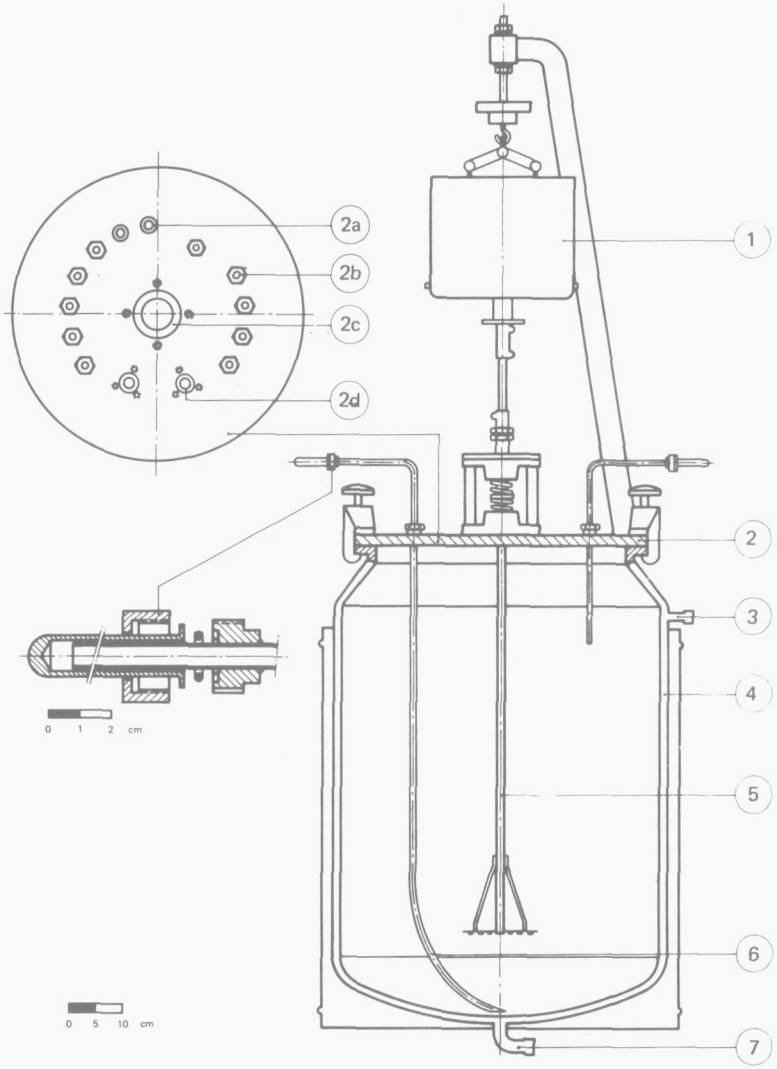


Fig. 3.15 200 litre D-container, vertical cross section.
1. Vibromixer; 2. head plate; 3. connection to double wall; 4. double walled stainless steel vessel; 5. Vibromixer shaft with impeller; 6. 8 mm tubing; 7. connection to double wall.
Head plate seen from above:
2a. standard pH-inlet (see Fig. 3.07),
2b. standard tube-inlet (see Fig. 3.06),
2c. central opening for Vibromixer shaft with membrane seal,
2d. openings for acid- and alkali-containers. In modified head plate two big filling openings are present instead.

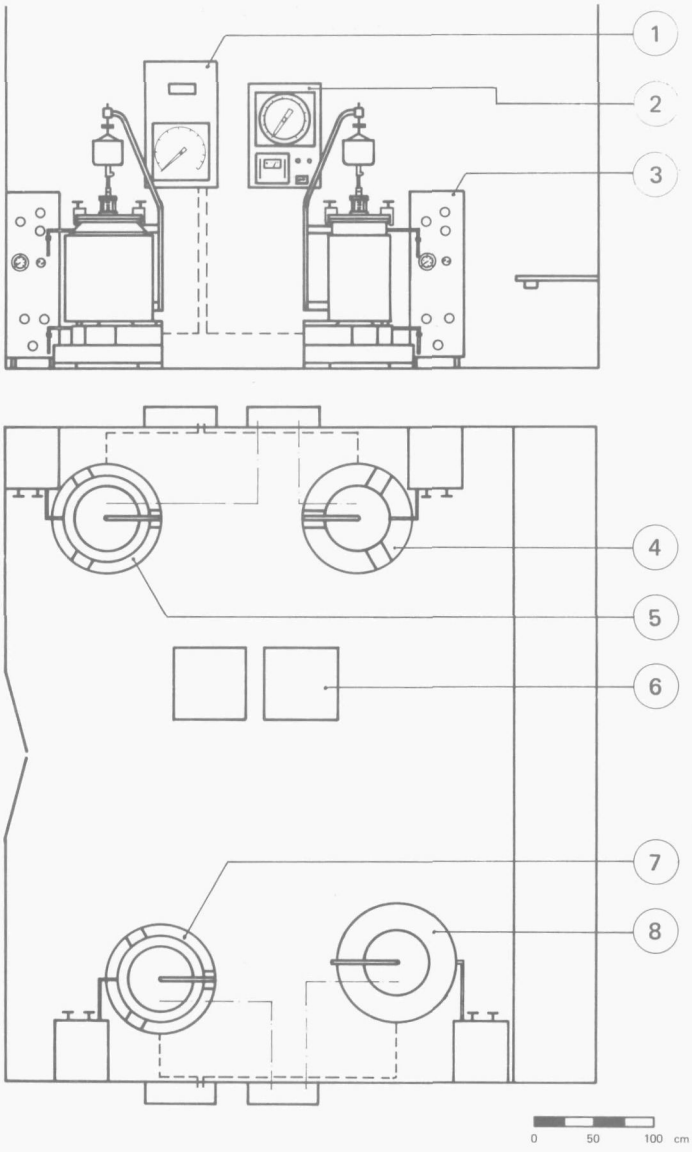


Fig. 3.16 Arrangement of mixing vessels and instrumentation in the vaccine mixing department of the Rijks Instituut voor de Volksgezondheid.
Top: looking towards a side wall. Bottom: seen from above.
1. Indicator and selector switch of electronic weighing apparatus.
2. Indicator/recorder for temperature and pH. 3. Panel with valves for steam, cooled water etc. to double wall of mixing vessel. 4. D-100 vessel. 5. D-200 vessel. 6. C vessels (transport vessels) to take up the mixed vaccine. 7. D-200 vessel. 8. D-400 vessel.

3.2 Measurement and Control

In the beginning of this chapter it has been stressed that much attention should be given to the measurement of physico-chemical variables. Virtually no cultivation process is performed without temperature control. pH has proven its importance from the moment good steam sterilizable electrodes became available; the same applies to the measurement of dissolved oxygen, for which we have contributed to the development, as will be shown below.

Some other variables that could be of importance and for which the principle of measurement is known, will be dealt with briefly, although no specific experience has been gained in our own experiments.

Measurement directly leads to the need for control although this has not been proven of advantage in all cases. For instance, the shift in pH during cultivation of *B.pertussis* gives a clear indication of the stage of growth (see Chapter 4, page 80 and Fig. 4.04 and 4.06). In this case pH-control has no advantage because it gives neither higher cell yield nor better protective activity and deprives us of the insight gained by following the pH-course.

The control systems will only be treated insofar they have determined the technical lay out of the systems, which have been constructed.

The simplest system of regulation is on-off control. It means that the controlling action is fully applied at all values below setpoint, and is fully stopped at all values above setpoint. Setpoint is the desired critical value of the variable. Opening or closing of a contact by mechanical, magnetic or optical coupling of a galvanometer needle to a switch is a common example.

Accuracy of control is not always achieved with the on-off method, especially in those cases where the effect of the controlling action is retarded. Retardation in the onset of effective control also means prolongation of the effect after the setpoint has again been reached. This results in so-called overshoot.

pH-control is an example where on-off control in almost all cases gives satisfactory results: the electrode follows the pH with negligible time delay, and the acid or alkali pump (or valve) immediately gives full control action after which pH shift follows, again without substantial time delay. The shift itself is restricted because of the good buffering capacity of most culture media. Only in the case of pH-control by varying the carbon dioxide concentration in the incoming gas is a delay to be expected, and proportional control will improve the result in most cases (cf. Section 3.2.2).

For temperature control and a f o r t i o r i for the control of dissolved oxygen, the situation is more complex. Heating elements all have their own

heat capacity causing delay. The time taken for the waterflow, coming from the heat exchanger (see Section 3.2.1), to reach the fermentor coil in our heating system is another reason for delay.

The situation is most complex in the control of dissolved oxygen: first there is the delay caused by the flow distance between the gas mixing chamber (see Section 3.2.3) and the fermentor; then there is the time needed to change the oxygen concentration of the headspace of the fermentor, and the time for oxygen to diffuse through the gas/fluid interface. Moreover, the buffering capacity of the fluid for oxygen is very low, demonstrated by the fact that a quick growing microorganism causes oxygen depletion in a few seconds, as the following calculation shows:

> The concentration of dissolved oxygen in water saturated with air at 37°C is 0.22 mM. Taking as a practical value for the oxygen consumption rate of a given bacterial culture 100 millimoles O_2/lh (cf. Fig. 3.21), this culture will consume all oxygen, contained in the fluid, in less than 8 seconds.

Where on-off control is unsatisfactory, proportional control is indicated. It means that the degree of control action of the variable is dependent upon the deviation from the setpoint. With still more sophisticated control the degree of control action is also dependent upon the rate at which the deviation is changing (derivative (D) control) and the time the deviation exists, (integrating (I) action). Only instruments exerting the last mentioned action have no proportional off-set; off-set means that the final value of the variable differs from the setpoint. This difference depends upon the experimental circumstances; only in cases where these circumstances are not varying to a great extent, and no great accuracy is needed, can proportional off-set be accepted.

The various instruments chosen in the panels developed in our laboratory will be discussed later on in this chapter. The following considerations helped us to make a choice in the jungle of measuring and control instruments available on the market:

1. *Transmittance of measuring c.q. control signal*
   The combination:
   sensor-indicator-controller, or
   sensor-recorder-controller
   is often needed.
   The normal arrangement has been, for years, to couple the controller mechanically to the indicator c.q. recorder, whether this is a galvanometric or a potentiometric instrument. The obvious disadvantage of

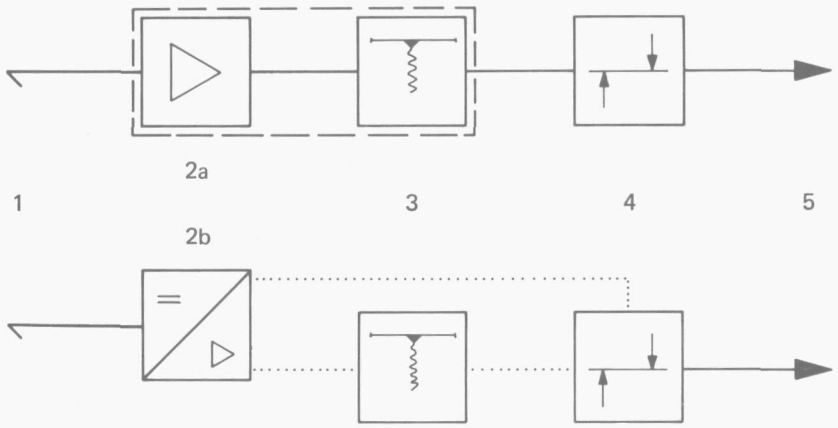


Fig. 3.17 Two principles of electronic control. Top: dependent upon indicator/recorder. Bottom: independent of indicator/recorder.
1. Measuring device (sensor). 2a. Amplifier. 2b. Converter. 3. Indicator/recorder. 4. Controller. 5. Control signal.
In the top scheme, 2a and 3 are usually built together. Dotted line: constant signalling current.

coupling to the moving indicator of the instrument is that it is liable to hysteresis and wear, and needs readjustment from time to time. In other words, control accuracy is also dependent upon the electrical and mechanical accuracy of the indicator or recorder. See schematic illustration in Fig. 3.17 (top).

Systems have recently been introduced which eliminate this drawback. Here the signal of the probe is converted to a current (called "standardized signalling current"; German: "eingeprägter Gleichstrom"). This current goes through a fixed range (of e.g. 0-20 mA in one system, and 4-20 mA in another), irrespective of the measuring range. In the loop formed by the output current indicators, controllers, recorders, and eventually switches, are taken up. In this way the functions are independent of one another, and no mechanical parts participate in the transmission of the signals. See Fig. 3.17 (bottom).

This new principle has been applied to the "Mentor" panel and in the "Oxy-Troll" panel, and also in the new series of "Paljas" panels.

2. *Control action*

Depending upon the variable to be measured and the accuracy desired, simple on-off control action or proportional control have been chosen; in the latter the necessity of added D (derivative) and/or I (integrating) functions was considered.

3. *Way of effecting proportional control*
   In all cases but one (oxygen control in industrial fermentors with positioning valve) the proportional control has been effected by varying the on to off ratio of the full strength control signal, known as time proportioning control. An example is the temperature control. In a cycle of e.g. 10 seconds the full capacity of 750 or 1500 Watts of the element is switched on for a part of this cycle, and completely off for the rest of the cycle. For small scale work it appeared to be the easiest solution, especially from the point of view of installation.

In the following sections the variables used in our work will be treated one by one. The control panels, containing the complete instrumentation will be shortly described subsequently.

3.2.1 TEMPERATURE

The optimal temperature for a cultivation process is not easy to define nor to determine accurately. Maximal yield of the desired antigen(s), whether this goes in parallel with cell yield or not, must be placed under primary consideration. The temperature of maximal yield may differ from the temperature of quickest growth i.e. shortest doubling time. Because yields cannot be determined with great accuracy for most processes under study, optimal temperatures are not known with greater accuracy than ± 1°C. Most processes in vaccine production are performed, not completely without arbitrariness, at 35°C. The fact that most data in the literature are obtained from cultures placed in an incubator (either cupboard or room) plays a role in this consideration. *R. Brouwer* (pers.comm.) has observed temperature differences in the order of 2-3°C between sites in commercial incubators.

Only in one instance is something known about temperature dependence of yield: if **Cl.tetani** is cultivated at 33.5°C instead of 35°C the yield of toxin is about 30 % lower.

The establishment of the process temperature is influenced by two features:

1. The reproducibility of the instrument i.e. the ability to repeat the same absolute value of the setpoint.
2. The accuracy of the control i.e. the deviation from the setpoint value on the meter.

Both values together should give a deviation from the desired absolute value that is less than 0.5°C. No process under our consideration shows a need for a higher accuracy.

In the choice of a heating system for our fermentors the maximal temperature at the surface of the heat exchanger (i.e. the coil) should not be so high that decomposition of cells or medium components at the surface is to be expected. Therefore a coil containing water was preferred above an electric element in the fermentor. A concomitant advantage of the system is that cooling in exothermic processes is achieved without complication, cf. the schematic representation in Fig. 3.18. In the system chosen it is necessary to use at least PD action control (cf. p. 39).

A thermocouple (Cu-constantan or Fe-constantan) has been chosen as temperature sensor. Thermocouples have the advantage over e.g. resistance thermometers of small dimension (in fact not thicker than the two wires together !) so that they are easily soldered in the tip of a closed 6 mm o.d. stainless steel tube. Both thermocouple combinations mentioned have a high V/temperature ratio which is important for accuracy. Although the output can be calculated theoretically, for maximal accuracy (better than 0.5°C) direct calibration against a standard thermometer has proven recommendable.

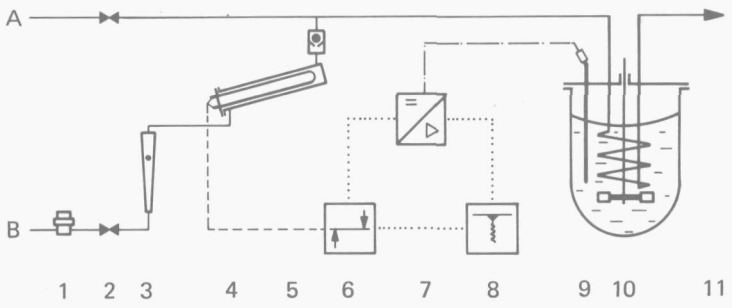


Fig. 3.18 Schematic representation of arrangement for sterilization, heating and cooling in fermentor.
A. Connection to steam mains.
B. Connection to water mains.
1. Differential pressure regulator. 2. Valves. 3. Flow meter. 4. Heat exchanger with electric element. 5. Backflow valve. 6. Temperature controller. 7. Converter (see Fig. 3.17). 8. Recorder. 9. Thermocouple. 10. Fermentor with coil. 11. Outlet to drain.

3.2.2 pH

pH-measurement in itself is no longer a problem with modern sterilizable combined electrodes. They have become available with increasing dependability over the last decade. Whereas it was formerly necessary to separate at least the reference electrode from the culture by an electrolyte bridge to prevent contamination and leakage of KCl into the culture, the modern electrodes are fitted with a small porous stone giving very little leakage and no contamination. Backflow of culture into the reference electrode reservoir, especially during sterilization at overpressure, can be prevented if the same (or a little higher) pressure is applied on the KCl reservoir. This requires a relatively complicated arrangement and we have found that with the Ingold combined electrodes closing of the KCl reservoir during heat sterilization and opening thereafter, and during cultivation, is an easier and still safe procedure.

As has already been concluded on p. 38, in pH-control accuracy is easily reached. The use of pumps with adjustable speed (e.g. of the peristaltic type) is preferred to valves, because we found the latter less dependable. Moreover, valves do not offer the possibility of varying the flow rate. If both pH-increase and -decrease are to be expected, two point control is necessary. A special case in which such a two point control is applied is described under Section 4.1.1. Schematic illustration of two point pH-control is given in Fig. 3.19.

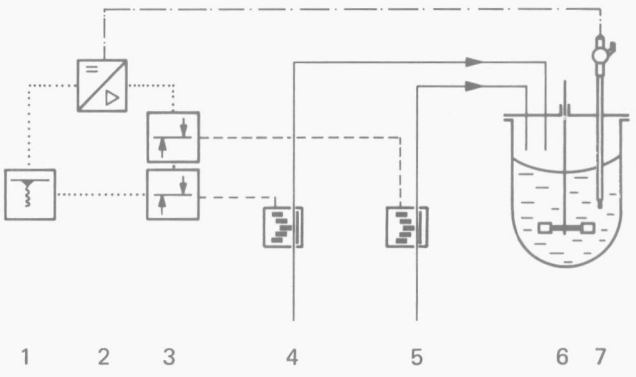


Fig. 3.19 Schematic representation of two point pH-control.
1. Recorder. 2. Converter (see Fig. 3.17) 3. Controllers. 4. Acid line from reservoir (not indicated) through peristaltic pump to fermentor. 5. Idem, for alkali. 6. Fermentor. 7. pH-electrode.

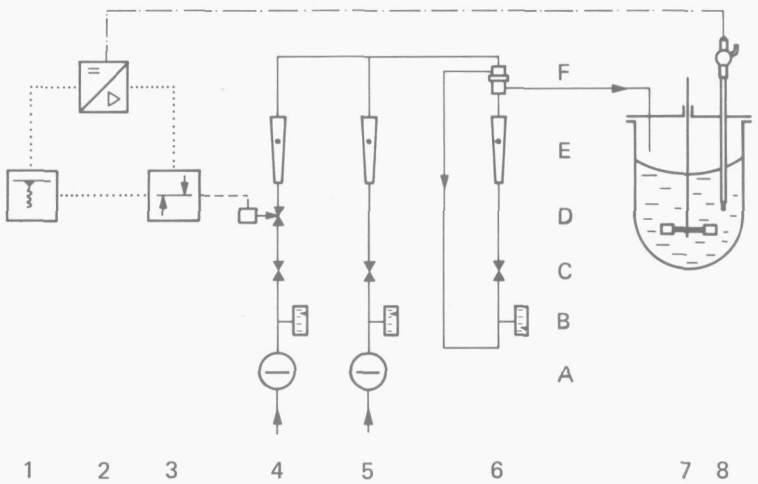


Fig. 3.20 Schematic representation of pH-control with carbon dioxide as the controlling agent.
1. Recorder. 2. Converter (see Fig. 3.17). 3. Controller. 4. CO_2-inlet. 5. Air inlet. 6. Combined gases. 7. Fermentor. 8. pH-electrode. A. Reduction valves. B. Manometers. C. Needle valves. D. Solenoid valve. E. Flow meters. F. Differential pressure regulator.

A more complicated pH-control consists of the addition of gaseous carbon dioxide to bicarbonate buffered systems (cf. *Telling* and *Stone*, 1964 and *Telling* and *Elsworth*, 1965). These systems are frequently used in cultivation of animal tissue cells. If such a system is present in a homogeneous culture of tissue cells the carbon dioxide concentration in the overlying gas phase will influence the pH. The control scheme is shown in Fig. 3.20. Because, in this case, carbon dioxide gas is added in the gasstream, a reaction lag occurs, comparable with the one in oxygen control. Therefore an on-off control will not always be accurate. Proportional control will then improve the results. A concomitant advantage of this system is that no extra electrolyte has to be added to the culture.

3.2.3 OXYGEN TENSION

Until the time that accurate measurement of oxygen tension in bacterial cultures became possible, a clear insight in the role of oxygen in bacterial cultivation was difficult to obtain.

Dependence of growth and other metabolic processes on the supply of oxygen has been known for long. The classical division into aerobic and anaerobic microorganisms in fact only indicated that there are microorganisms on which the presence of oxygen has a poisoning effect. For anaerobic microorganisms the pO_2 will be zero. For aerobic microorganisms there will exist an optimal pO_2 for production of cell material and one for production of certain metabolites, and these two optima do not necessarily coincide.

In industrial fermentations air supply is an important factor in consideration of production costs. The aeration efficiency is the guiding parameter: it indicates the amount of oxygen taken up per unit of culture volume per unit of time under defined conditions of vessel dimensions, filling volume, air flow rate, stirrer speed and method of aeration (vortex or sparger and, in the latter case, type of sparger). Fig. 3.21 shows the results of the determination of the aeration efficiency, determined by the method of *Cooper* et *al.* (1944), as a function of stirrer speed and air flow rate, in a 5 litre vessel of the Bilthoven Unit. In this method the fermentor is filled to the required

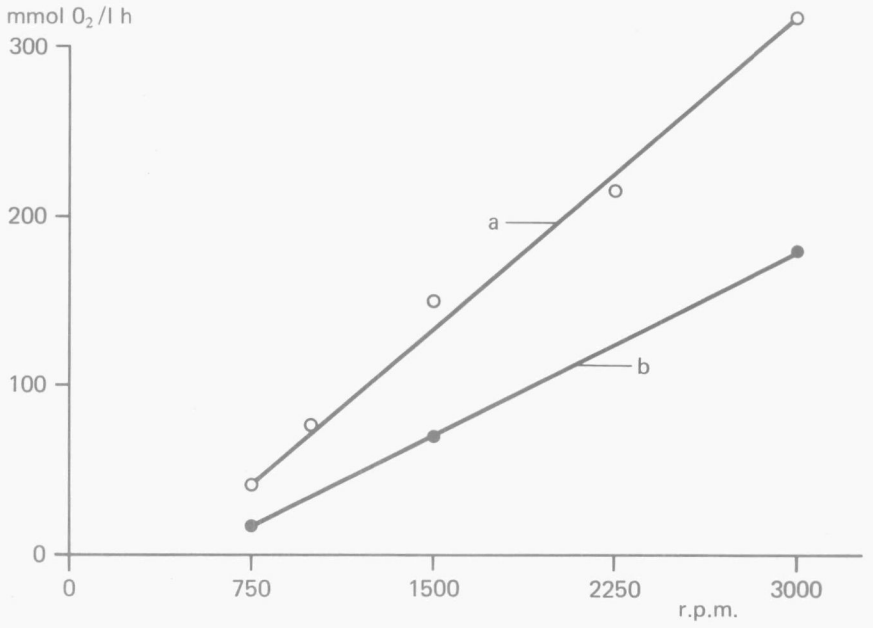


Fig. 3.21 Aeration efficiency as a function of impeller speed and air flow rate. Culture volume 3 litres.
a. air flow rate 10 l/min. b. 3 l/min.

volume with a solution of Na_2SO_3, with $Cu^{\cdot\cdot}$ ions as a catalyst. The oxygen concentration in the fluid is thus always kept at zero, because the inferface between fluid and microorganism is absent. Hence the method has been severely criticized on theoretical grounds (*Herbert*, 1961). It will however give at least an impression of the influence of the parameters mentioned and is still in general use for that purpose.

It is obvious that a culture grown under conditions giving a certain aeration efficiency (as determined with the method of *Cooper* et *al.*), will give no information about the actual concentration of oxygen in the culture.

In the theory of growth kinetics of a bacterial culture the growth rate is assumed to be dependent upon one nutrient, designated the limiting factor. Maximal growth rate is only achieved if all nutrients are supplied in excess. Oxygen is in many cases the limiting factor. Oxygen is not, as other nutrients normally are, added at the beginning of the culture but supplied continuously during cultivation. It may restrict growth in a special way: the addition of equal amounts per unit of time gives rise to a linear instead of a logarithmic growth pattern. If growth rate would be constant as would happen with excess oxygen, growth is expected to proceed logarithmically, as follows directly from the equation:

$$\mu = \frac{1}{N} \frac{dN}{dt}$$

where μ is the exponential growth rate, N the number of bacteria per unit of volume, and t the time. If growth is logarithmic, the oxygen consumption is increasing logarithmically, As soon as the oxygen consumption reaches the maximal transfer rate, under the circumstances of the culture, the oxygen consumption rate becomes constant.

In practice linear growth is indeed observed, thus indicating scarcity of oxygen. The actual oxygen concentration in the culture is, in this case, almost zero. According to *Herbert* (loc. cit.), the limiting concentration of oxygen for most (aerobic) organisms is in the order of 10^{-3} mM; this implies that oxygen limitation is normally not to be expected above 1 % air saturation in cultures at 37°C.

Only if the supply of oxygen is such that a certain concentration of dissolved oxygen is built up in the culture, and maintained, by means of pO_2-control (see p. 54 sqq.), is a new situation created in which dependence of metabolism upon the oxygen concentration can be studied.

The determination of the oxygen concentration (calculated commonly as oxygen partial pressure, or oxygen tension, and expressed in mm Hg, saturation with air being around 150) has been made possible by the

development of probes, that can be steam sterilized together with the contents of the fermentor. The development by *Van Hemert, Kilburn, Righelato* and *Van Wezel* (1969) of such an electrode that has been accepted in several other laboratories will be described here in detail.

Although measurement of dissolved oxygen in bacterial cultures is no problem theoretically, there was until recently no practical technical solution which would make the measurement of oxygen pressure a routine procedure. The situation was comparable with the state of pH measurement in bacterial cultures a few decades ago.

There was a need for a sturdy, steam sterilizable probe, which once assembled and put into place, could be easily calibrated and handled by the laboratory technician.

The oxygen electrode to be described here has been developed with these conditions in mind. Its components can be easily made in a workshop from normally available materials, and are of standard design permitting complete interchangeability.

It was felt that the silver/lead galvanic element separated from the culture by a thin membrane, permeable to gases (*Mancy* et *al.*, 1962; *Mackereth*, 1964) was the most promising principle to use as a starting point. This system has a constant very low zero current and gives a high current response to oxygen provided its dimensions are properly chosen. Since no external voltage need be applied, the electrical circuitry is simple.

The *Mackereth* electrode (type A 15 A, marketed by Electronic Instruments Ltd.) was used by *MacLennan* and *Pirt* (1965). They suggested that the electrode could be steam sterilized if a FEP-Teflon membrane was used instead of a polythene one, but this proved to be impossible (*MacLennan*; pers. comm.). We found that ethylene oxide sterilization is the only method practicable; it was the method used by *MacLennan* and *Pirt* (1966) and by *Flynn, Kilburn, Lilly* and *Webb* (1967). These last authors also recommended several changes in the design in order to improve the stability and the electrical connections.

An other design using the same principle is that of *Johnson, Borkowski* and *Engblom* (1964; see also *Borkowski* and *Johnson,* 1967), who used an open electrolyte reservoir, which is a prerequisite for dependable heat sterilization. Because of its small dimensions the output current is only in the order of 0.05 μA/mm Hg. Therefore an amplifier is needed, or a high resistance has to be put in the output circuit, which can reduce the potential in the galvanic element impairing linear response. Moreover, since the body consists of an open glass tube it is a rather vulnerable instrument, only suitable for laboratory experiments.

An other way to achieve sterilizability is to bring the probe, after sterilization, into a tube closed with an extra glassfibre-reinforced silicone

membrane (*Ring* et *al.*, 1969). The principle of this electrode is of the so-called *Clark* cell type (polarographic type; see *Gleichmann* et *al.*, 1960). This principle has certain disadvantages, if compared with the galvanic type (high zero current; needing a more complicated electronic instrument). Moreover, the use of a double membrane (one on the electrode itself, a second one on the sterilizable tube), will undoubtly impair the rate of response to step changes in pO_2.

In the following description of the electrode, numbers refer to parts in Fig. 3.22, unless otherwise stated.

STERILIZABILITY

In order to make the silver/lead cell steam sterilizable it was found that a construction was needed in which the electrolyte is able to expand. However difficulties still occurred when the cell with electrolyte was brought to the high temperature required for sterilization. Therefore it was decided to sterilize the assembly filled with distilled water and to replace the water with electrolyte after sterilization. The electrolyte found most satisfactory is half saturated potassium bicarbonate. In the design, measures have been taken for easy replacement of the fluids. Because the electrolyte has the higher density, and the probe is normally used face down, the ingoing tube (1) is used to bring the fluid to just above the silver cathode (9). The outgoing tube (Fig. 3.23) is connected with the highest point of the reservoir.

INTERIOR OF THE CELL

The reservoir itself (3) is made of nylon. Because this material is easily deformed, especially by changes in temperature and humidity, it is encased in stainless steel (4). The lead anode (11) consists of a strip of 0.1 x 16 x 400 mm, wound to fit into the nylon. Spacing between the windings is secured by little protuberances on the surface of the lead. The anode is soldered within the electrolyte space directly to a lead wire; this arrangement ensures electrical reliability, while the presence of a bare solder connection is apparently not detrimental to the operation of the electrode.

The silver cathode (9), of about 400 mm^2 surface, is separated from the lead by a disc (7) of porous PVC (Vyon; $^1/_{16}$ in. thick). There are holes of 1 mm diameter to bring the electrolyte to the silver surface adjacent to the membrane (8). This surface should be roughened, giving just visible scratches (cf. Performance).

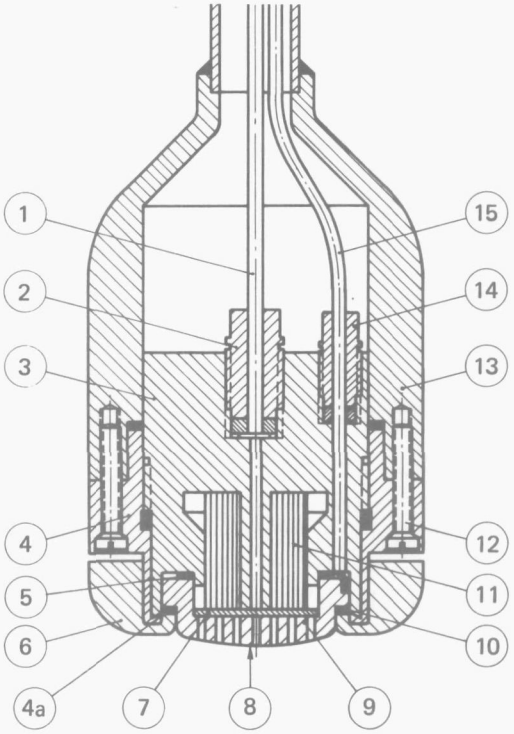


Fig. 3.22 Cross section of oxygen electrode, laboratory model. Connection of the silver cathode is shown. Numbers are referred to in the text, except
12. screws;
14. standard fitting.

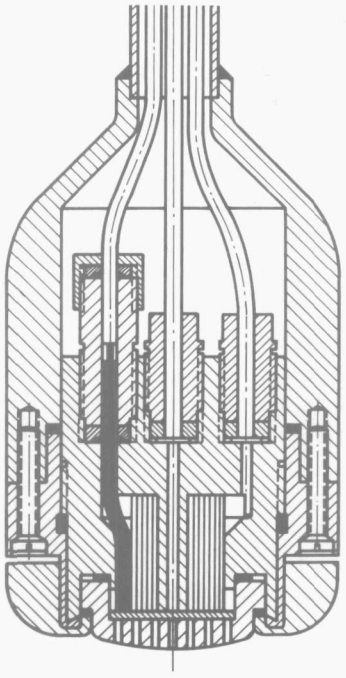


Fig. 3.23 Cross section of oxygen probe, turned 90° compared with Fig. 3.22. Connection of the lead anode is shown.

PLACEMENT OF THE MEMBRANE

Cylindrical membranes have the disadvantage that they are difficult to seal to the electrode; moreover, the required size of plastic tube material is not always available. On the other hand a flat membrane, if applied over the end of a tube, gives rise to folds and hence leakage. A special system to apply a flat piece of membrane, about 50 x 50 mm was developed; it is first secured by a stainless steel ring (6) and then stretched over the cathode, when this ring is screwed down by four bolts. An O-ring (10) forces the membrane down onto the outer rim of the silver cathode, completely sealing the electrode, and fixing the membrane firmly over the lens-shaped surface of the silver. This guarantees a constant minimal distance between membrane and silver, necessary for a rapid and reproducible response to the oxygen tension outside the membrane.

INTERNAL CONNECTIONS

There are two electrical and two tubing connections. The former consists of Teflon insulated copper wire (15), the latter of Teflon tubing (1) connected to the channels in the nylon body by standard fittings (2). One electrical wire is soldered at a place outside the electrolyte into the silver cathode; the other is soldered against the end of the lead wire of the anode at a place outside the electrolyte and occluded between two small O-rings (Fig. 3.23).

HOUSING

The main part of the housing is cylindrical, approximately 46 mm outer diameter, and made of stainless steel (13). There are two models: for the laboratory type the cylindrical part tapers off into a welded connection with a stainless steel tube of 12 mm outer diameter. This tube fits snugly in one of the electrode inlets of the fermentor described, next to the combined pH-electrode as shown in Fig. 3.25. Moreover, as 12 mm is a standard size for several marks of pH-electrodes, the oxygen probe will also fit in many vessels of other origin.

In the second type (Fig. 3.24) the cylindrical part is, with only a slight change in diameter, welded to a stainless steel tube, which in turn is welded to a standard flange. This provides an easy arrangement for installation through the top or the side wall of an industrial fermentor.

The two electrical and two fluid leads are brought through the stainless steel stem of either 10 or 32 mm inner diameter to the exterior.

ASSEMBLY

The lead anode (11) is soldered into position in the nylon body (3) which has already been screwed in its stainless steel casing (4). The Vyon disc (7) is

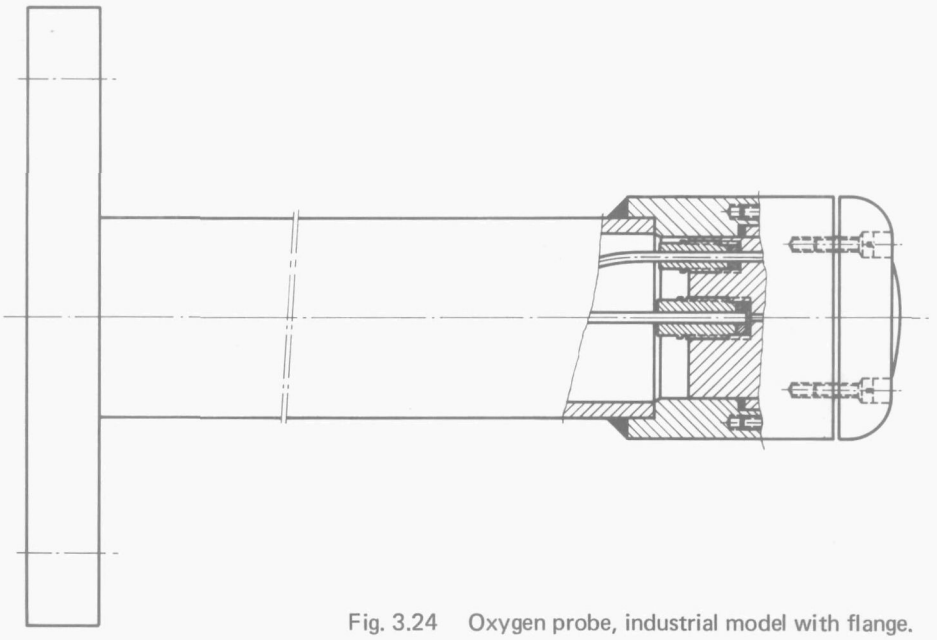


Fig. 3.24 Oxygen probe, industrial model with flange.

laid over the anode, and the silver cathode (9) is lightly pressed down on the deepest O-ring (5). Then electrical and tubing connections are fastened tightly in the connectors. This assembly is bolted to the main body (13) of the probe. For the placing of the membrane (8) the electrode is kept vertical, e.g. in a laboratory clamp, face upwards. The membrane is laid flat over the silver surface. The stainless steel ring (6) is pressed cautiously over the outer ridge (4a) of the reservoir casing, but is not forced down. The O-ring (10) is laid on the membrane, just under the inner ridge of the stainless steel ring. Finally the ring is screwed down evenly, stretching the membrane over the silver surface.

OPERATION

Distilled water is injected through the central channel (by placing a syringe in the appropriate tube), until it flows over from the other tube; the reservoir is subsequently rinsed with several volumes of distilled water.

The probe is steam sterilized with the fermentor, only the tube connected with the central channel being closed. After cooling, half saturated potassium bicarbonate is brought through the central channel, until about 20 ml of fluid have escaped from the other channel. Then both tube ends are closed with a tube clamp.

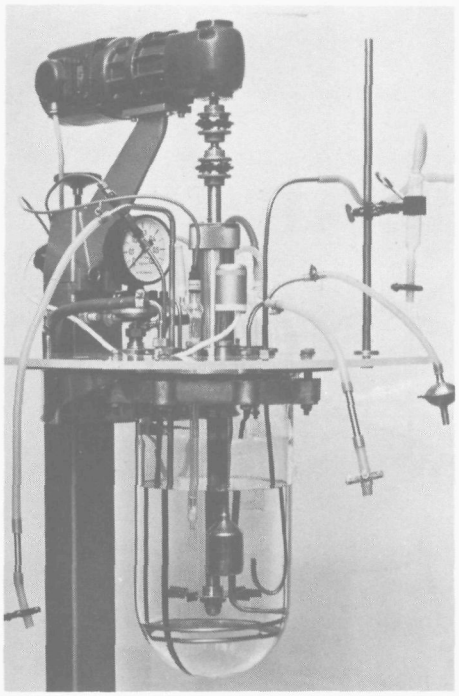


Fig. 3.25. 10 litre fermentor with O_2-electrode

For equilibration the electrode is left in the fermentor while stirring, in equilibrium with air until the reading is stable. Although this normally takes about two hours, in practice we always leave the electrode overnight for this purpose.

Electrical connections are made across a variable resistor of 0-25 Ω. The potential drop across the resistor is measured preferably on a 0-1 mV recorder, because a low span allows the use of a low external resistance, which speeds the response to changing oxygen concentrations (*Mackereth,* 1964; *MacLennan* and *Pirt,* 1966).

Calibration is normally performed by feeding mixtures of nitrogen and air through the fermentor which is filled with water or medium. These mixtures are made by mixing streams of known flow rate of both gases.

PERFORMANCE

This oxygen electrode has been used extensively in bacterial and animal cell cultivations including continuous cultures of over 4 weeks' duration, without appreciable change in calibration values. In addition, experiments to check the performance have been carried out and are recorded here.

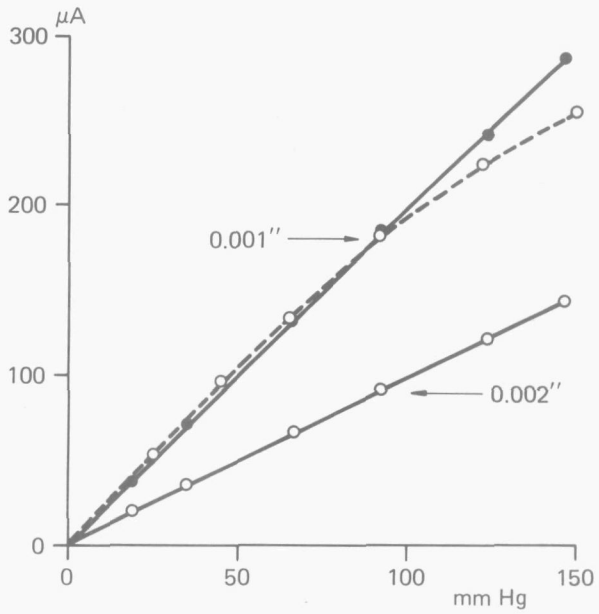


Fig. 3.26 Response of O_2-electrode at different oxygen partial pressures. External resistance 1.21 Ω. Temperature 33.7°C.
Dotted curve: insufficiently roughened cathode. Thickness of FEP-Teflon membrane mentioned in the figure.

The response depends upon membrane thickness, and is of the order of 2 μA per mm Hg for the 0.001 in. (= 0.025 mm) membrane, and 1 μA for the 0.002 in. (= 0.050 mm) membrane, both of FEP-Teflon. In Fig. 3.26 typical calibration curves for these two thicknesses are given. The oxygen partial pressure of the mixtures applied are determined by reading the oxygen percentage on a paramagnetic analyzer (Magnos, Hartmann and Braun); the reading is converted to partial pressure, taking into account the overpressure and the water vapor pressure in the vessel and the barometric pressure.

For the thinnest membrane two curves are shown: the only difference is the roughness of the silver surface, the rougher surface showing better linearity. This suggests that the membrane is stretched so tightly over the surface that too thin a layer of electrolyte is present at the interface, resulting in a lower relative response at higher oxygen tensions. Small channels should be formed by proper roughening of the cathode surface.

Fig. 3.27 shows the response time using the 0.001 in. membrane when the

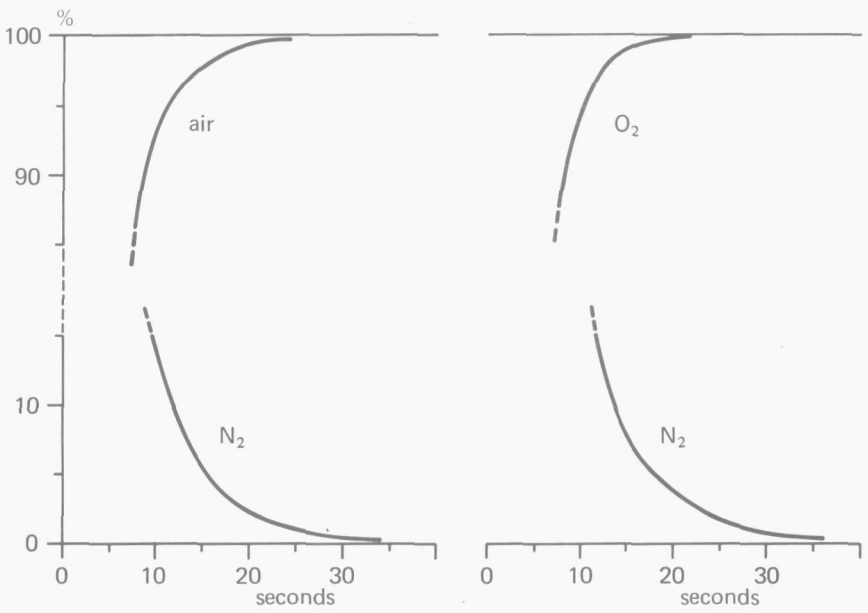


Fig. 3.27 Rate of response of electrode to sudden change in oxygen tension, effected at time O. 100 % = equilibrium current with air c.q. oxygen (approximately 250 c.q. 900 μA). 0 % = equilibrium current with nitrogen. External resistance 1.21 Ω. Temperature 25°C.

electrode is brought suddenly from nitrogen to air, and from nitrogen to oxygen, and vice versa. The 90 % response time is less than 15 seconds in all cases, which is consistent with the response rate required in bacterial cultures.

The electrode has proven to survive at least 25 cycles involving sterilization, filling with electrolyte, cultivation of 1-7 days, refilling with water, sterilization, etc. The decrease in calibration value during one culture depends a.o. upon the electric current during the culture. This decrease is in many cases negligible, and always less than 1 %.

OXYGEN CONTROL

As has been mentioned already in section 3.2 (p. 39) the control of oxygen partial pressure in a bacterial culture is more difficult than either temperature or pH-control in the same environment. The main reason is that the buffering capacity of a culture in respect to oxygen is very low: a quick growing culture will be depleted of oxygen a few seconds after cutting off the supply.

This behaviour poses special problems for the device intended to keep the oxygen partial pressure in a culture constant. On-off control would cause intolerable overshoot, so proportional control is a necessity.

There are three principally different methods to adjust oxygen uptake to a culture. In order to illustrate these methods the following general equation is given:

$$N_V = K_l \cdot a \, (C^* - C_l)$$

in which N_V is the oxygen solution rate, C^* the oxygen concentration of the culture in equilibrium with the gas phase, C_l the concentration of oxygen in solution in the culture and $K_l \cdot a$ the volumetric mass transfer coefficient, dependent upon the conditions of cultivation. It is clear that, if C_l has to be maintained in a situation of increasing oxygen demand by the culture fluid, $K_l \cdot$ or C^* has to be increased; hence the three methods:

1. Increase of agitation (stirrer speed), which increases the transfer coefficient $K_l \cdot a$.
2. Increase of air flow rate. If air is sparged into the liquid, the main effect will come from increase of $K_l \cdot a$, although a higher gas pressure at the moment of escape will contribute to a higher C^*. When vortex aeration is applied, the dependence of the aeration efficiency on the flow rate, as shown in Fig. 3.21, is clearly present; here change of $K_l \cdot a$ will almost exclusively be responsible.
3. Increase of the oxygen content of the gas offered to the culture.

The latter method, although being the most sophisticated and accurate one, is only applicable on small culture volumes. In industrial fermentations the cost of oxygen gas is prohibitory.

The principal arrangement for the methods 1 and 2 is shown in the Figures 3.28 and 3.29. In both cases a positioning motor, receiving the controlling signal changes the position of a speed control on the stirrer motor c.q. a positioning gas valve. A special control instrument for this industrial application has been developed (Oxy-Troll 19 I). For the processes described in the next chapter, however, whether on 7 or on 40 litre scale, the control arrangement with variation of the oxygen concentration in the gas (3) was preferred.

Fig. 3.30 shows the arrangement for this control method. The pressures of the three gases are reduced until the three manometers show the same pressure (e.g. 1.5 kgf/cm^2) when there is no flow. The valves C are adjusted thereafter so that the three flow meters show the same flow rate. The valve in the total flow line serves to set the final rate which is, by virtue of the differential pressure regulator, independent of the fact that one, two or three gases are transmitted.

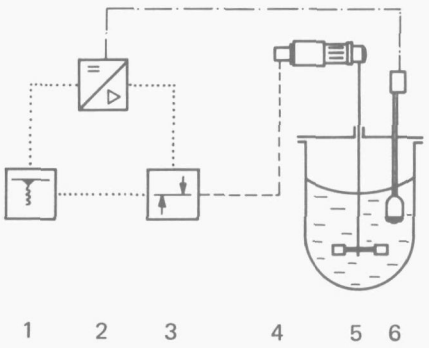


Fig. 3.28 Schematic representation of pO_2-control by means of varying the stirrer speed.
1. Recorder. 2. Converter (see Fig. 3.17). 3. Controller.
4. Stirrer motor with speed variator, connected with positioning motor. 5. Fermentor.
6. Oxygen probe.

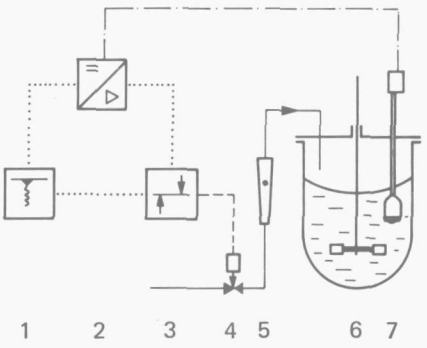


Fig. 3.29 Schematic representation of pO_2-control by means of varying the air flow rate
1. Recorder. 2. Converter (see Fig. 3.17). 3. Controller.
4. Positioning valve. 5. Flow meter. 6. Fermentor.
7. Oxygen probe.

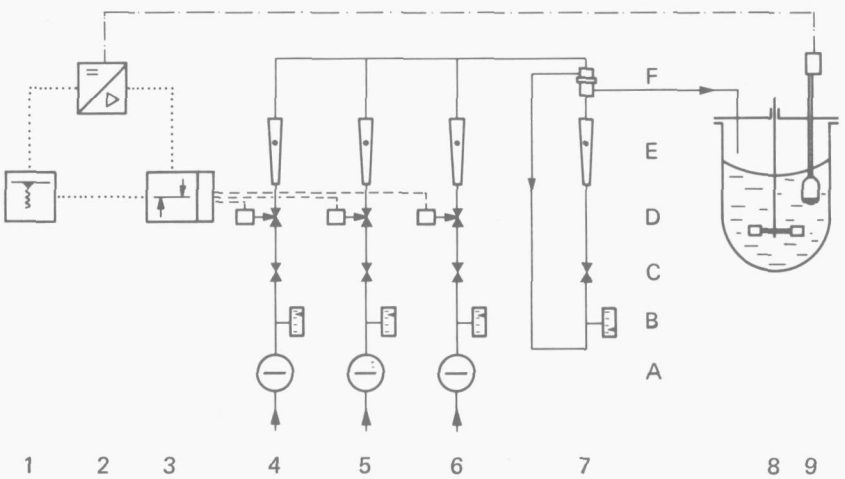


Fig. 3.30 Schematic representation of pO_2-control by means of varying the gas composition.
1. Recorder. 2. Converter (see Fig. 3.17). 3. Controller with selector switches for combination of gases (see Table 3-01). 4. Oxygen line. 5. Air line.
6. Nitrogen line. 7. Combined gases. 8. Fermentor. 9. Oxygen probe.
A. Reduction valves. B. Manometers. C. Needle valves. D. Solenoid valve.
E. Flow meters. F. Differential pressure regulator.

Besides the fact that this control system gives a constant flow rate, permitting calculation of gas consumption and evolution by means of oxygen and carbon dioxide analyzers, it is the most accurate one. Table 3-01 shows how the interval of oxygen concentration can be chosen. The more adapted the interval is to the given circumstances, the better will the control be at a certain value of the pO_2 in the culture.

Fig. 3.31 shows the instrument with which this oxygen control method is performed. By means of a set of seven switches the three gases can each be made to flow permanently, while oxygen and air can be commanded when the oxygen value is below setpoint, and air and nitrogen when above setpoint.

Examples of control of pO_2 and the usefulness in some cases will be given in Chapter 4.

Table 3-01 Ranges of O_2-concentration, obtained with several O_2-control combinations. Percentages are based on the assumption that flow-rates are equal.
Low means: gas added below setpoint.
High means: gas added above setpoint.
Perm. means: gas added permanently.

Combination no.	O_2		AIR			N_2		% O_2 in gas	
	low	perm.	low	perm.	high	perm.	high	max.	min.
1	x			x				60	20
2	x				x			100	20
3	x					x		50	0
4	x						x	100	0
5	x			x		x		40	10
6	x			x			x	60	10
7	x				x	x		50	10
8		x			x			100	60
9		x					x	100	50
10		x	x				x	60	50
11		x		x			x	60	40
12		x			x	x		50	40
13			x			x		10	0
14			x				x	20	0
15				x			x	20	10

(Courtesy N.V. Apparatenfabriek van Doorn, de Bilt)

Fig. 3.31. "Oxy-Troll"

3.2.4 OTHER VARIABLES

There are a few other variables whose consideration could be of advantage in certain culture processes, and for which a principal control method is available. Electrodes for the measurement of several ions, such as chloride, nitrate and calcium, exist nowadays. The electronic instrument is of the same type as for measuring pH. As far as we know, they are not steam sterilizable but could be sterilized chemically. The change in ion concentration will only be of interest in special cases: in continuous culture where dilution and change of medium supply can occur, the ion electrode may prove its use.

The possibility of measuring glucose concentration with an electrode has been brought forward by *Updike* and *Hicks* (1967). They make use of an

oxygen electrode, comparable with the one described by us. For this purpose the membrane is covered with a thin layer of a gel containing glucose oxidase. The diffusion flow of oxygen through the membrane to the oxygen electrode is reduced because oxygen is used according to the following equation:

$$\text{glucose} + O_2 \rightleftharpoons \text{gluconic acid} + H_2O_2$$

Difficulties include the need for a reference electrode indicating the pO_2 in the culture fluid, and the fact that the enzyme is heat labile. The principle, however, is so promising, also for determination of other compounds, for which an enzyme with the same reaction type exists, that work to develop an "enzyme electrode" has started in our laboratory.

Of more general application are the attempts that have been made to measure opacity directly and continuously in the culture. Although the method to correlate the extinction of transmitted light with germ count has its limitations (as discussed on p. 65), it is a method that is still used generally. The greatest difficulty in constructing a measuring cell in the culture fluid is the fact that the cuvette surfaces will gradually become covered. The existing systems provided with mechanical wipers lack simplicity and we consider them as undependable (cf. *Blachère* and *Lamart*, 1969). Very recently a different approach has been made (*New Brunswick*

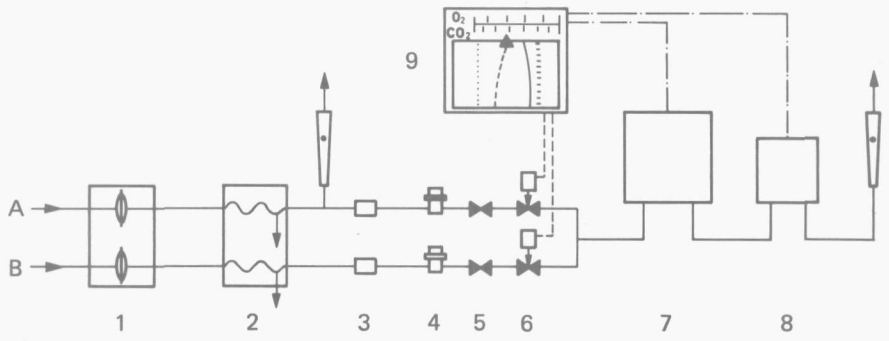


Fig. 3.32 Analyzer for oxygen and carbon dioxide gas; schematic representation of gas flow and electric arrangement.
1. sterile filter (heated at 70°C to avoid water condensation); 2. condensor; 3. pump; 4. differential pressure regulator; 5. needle valves; 6. solenoid valves; 7. CO_2-analyzer; 8. O_2-analyzer; 9. 4 point recorder, steering also the change from channel A to B with valves 6.
A. Gas outlet of culture. B. Sample of gas inlet of culture.

Sci.Cy.). Here the light extinctions through two tubes, of known, differing inner diameter are subtracted thus eliminating the extinction caused by covering of the surface. The principle is only valid if the deposit in both tubes is the same.

The measurement of the oxygen and carbon dioxide concentrations in the gas phase has no direct bearing on any variable of the culture process. Nevertheless, the knowledge gained may contribute to the elucidation of the carbohydrate metabolism. A set up developed by us measuring both variables, using existing gas analyzers, is shown in Fig. 3.32. It measures, with intervals of 8 minutes, the concentrations of the two gases, both in the inlet and in the outlet of the culture. Calibration inaccuracies of the analyzing systems are thus levelled out to a great extent. When the gas flow rate through the culture is constant, which is technically possible (also in case of oxygen control cf. 3.2.3), an oxygen and carbon dioxide balance can be calculated. A discussion of the necessary calculation is given by *Fiechter* and *von Meyenburg* (1968). The method proved to be useful in the study of the metabolism a.o. of **C.diphtheriae** (cf. section 4.2.1).

Measurement of redox-potential (eH) has never been applied in our studies. Although used by several workers, for the last 40 years, the belief in the importance of this variable, as a function of culture time, has gradually been lost. It is undeniable that the eH of a growing culture always tends to go to more negative values. But a bacterial culture contains many undefined redox systems, including the cells themselves, out of equilibrium, and the output of the probe (a naked platinum wire used in conjunction with a calomel reference electrode) may be a complex function of any of these systems. The response will be completely unpredictable, except on an empirical basis under strictly defined conditions (*Harrison,* 1971). In simple systems, such as a phosphate buffer, eH goes up and down with pO_2 (*Jacob,* 1970 a; 1970 b). Most investigators, however, prefer nowadays the measurement of dissolved oxygen tension, as giving more meaningful information. Perhaps, in the cultivation of anaerobic microorganisms, there may be a – strictly empirical – application for eH measurement.

3.2.5 INTEGRATED CONTROL PANELS

It has been our opinion that as soon as a certain technical arrangement has proven its usefulness and is to be used repeatedly, one should proceed from the more or less spontaneous bench set up to an arrangement, called "black box" i.e. a compact arrangement of all components within a frame. Almost

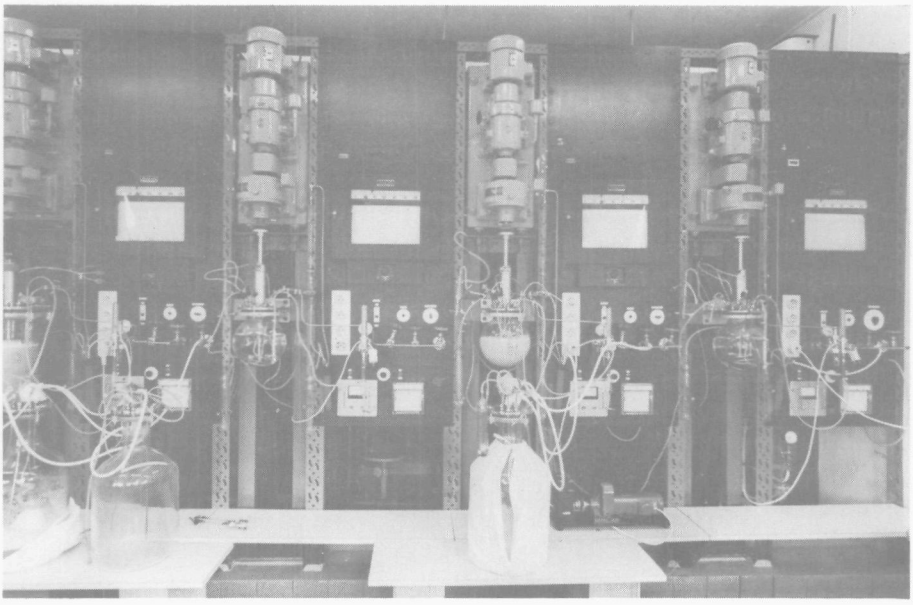


Fig. 3.33. The first "Bilthoven Unit"

always such an arrangement saves space, and only exposes meter and switch surfaces and valve handles. It adds to the ease of handling of the apparatus, and of inspection of the process. An other advantage of using a "black box" is that sturdier material and better connections will be used for pipelines and electricity, giving considerably more safety and dependability to the arrangement. If, finally, the external connections are made standard in the case of a single purpose and different in case of comparable but different purposes, an instrument is obtained for which a simple, unequivocal instruction can be drawn up.

The control panel serving the Bilthoven fermentor (in many instances two fermentors) has gone through several stages of evolution. From the first apparatus, consisting of a vertical plate onto which the fermentor was also fastened (see Fig. 3.33 cf. *Van Hemert,* 1964b), there was a progression to a movable cupboard (resembling in form a pianoforte), with two fermentors on their own movable supports (cf. Fig. 3.34). A simplified version of the latter, called the "Paljas" Unit, gives maximal compactness and only contains the strictly necessary features. The fermentors are the same as those used for the larger panel. It is mainly this unit, that is in use in several other laboratories (see p. 160) for the production of bacterial vaccines.

Recently a new series of "Paljas" Units has been developed, making use of the newer electronic system with standardized signalling current, described on p. 40.

"PALJAS-N"

For two Bilthoven fermentors with temperature and pH-control and pO_2-control (for connection to external controlling systems). Temperature, pH and pO_2 of both fermentors are recorded.

"PALJAS-S"

For one fermentor, having, in addition to the above, built-in pO_2-controlling organs (as depicted in Fig. 3.30). See Fig. 3.35.

"PALJAS-T"

For one stainless steel vessel of 120 or 250 litre culture volume. Specially adapted for cultivation of **Cl.tetani.** There is a built-in incinerator for the outcoming gas. (See Fig. 4.34).

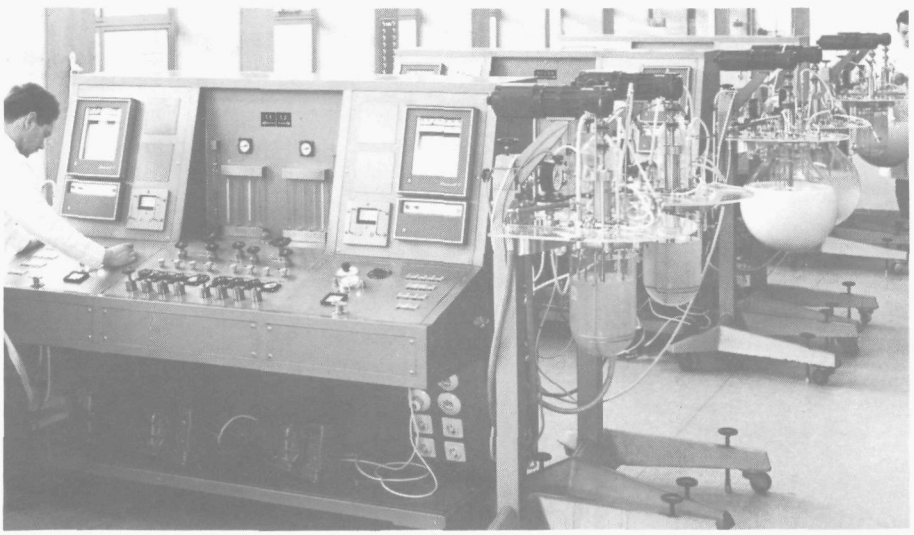
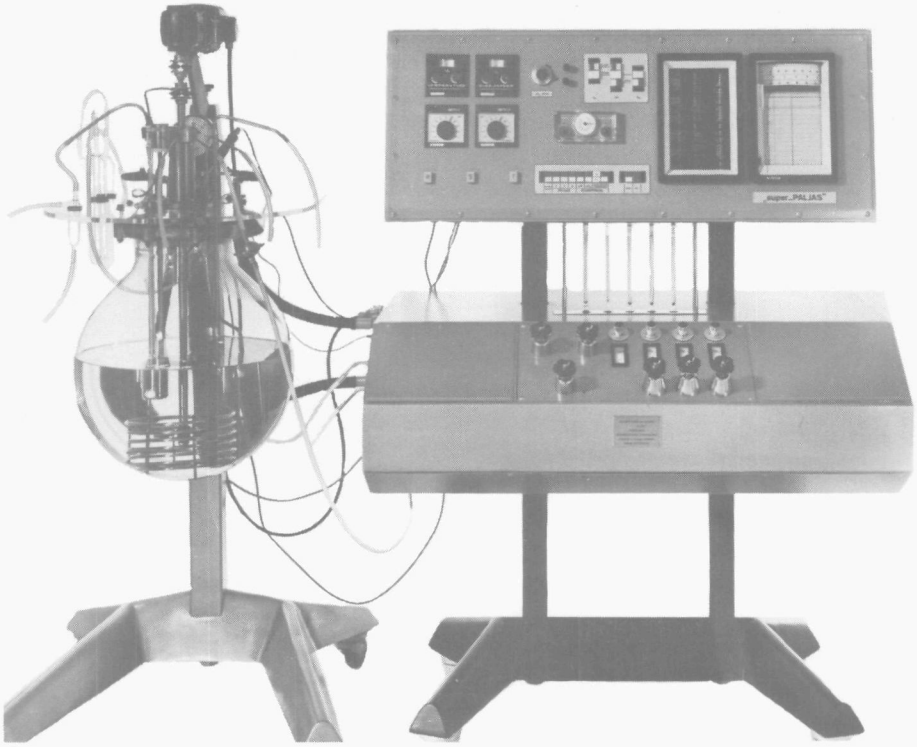


Fig. 3.34. The new "Bilthoven Unit", in use for bacterial vaccine production at the R.I.V.

"PALJAS-M"

Resembling the S-type, but adapted to serve one stainless steel fermentor of 350 or 500 litre, with temperature, pH- and pO_2-control and recording.

The newest version, that can be considered as replacing the first large movable unit, (cf. Fig. 3.34), is the "Mentor" Unit shown in Fig. 3.36. Serving again two movable fermentors, it contains continuous recording and control for temperature, pH and oxygen tension as well as a possibility to add a fourth variable.



(Courtesy N.V. Apparatenfabriek van Doorn, de Bilt)

Fig. 3.35. "Paljas-S" control unit with 50 litre fermentor

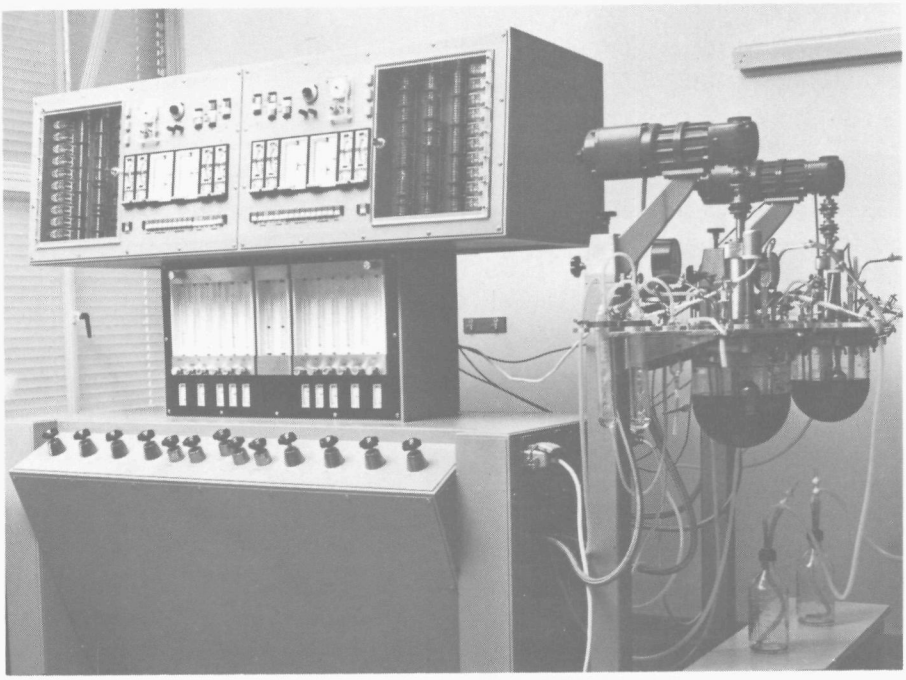


Fig. 3.36. The "Mentor" control unit

3.3 Cultivation Techniques

In the introduction to this chapter (p. 17) the arguments for the choice of homogeneous cultivation have been given, together with a discussion of the sense of the word homogeneous in this context. The batch cultures to be described in Chapter 4 may be considered, to the best of our knowledge, as homogeneous, in the sense used. If a culture, besides being homogeneous in this sense, is also in the steady state of a continuous culture process, an other dimension is added to the homogeneity: that of time. The reasoning that led us to the conclusion that, once the optimal conditions in a batch culture have been found, a homogeneous (in the spatial sense) culture offers the possibility to establish these optimal conditions in every part of the culture, is, mutatis mutandis applicable to a steady state continuous culture: if a culture can be held, in terms of time, in the best condition for the fulfillment of the required task, this is to be preferred to a batch culture in which the optimal conditions only exist during a limited time span.

This reasoning is of theoretical origin; the reasons why in practice batch culture is frequently chosen will be discussed in section 3.3.2.

For the evaluation of the density of bacterial cultures, comparison of the opacity with that of a standard has been common practice in bacteriology for many years. Although debatable from a theoretical point of view, the method is much quicker than cell counting methods, performed under the microscope or on plates, or determinations of bacterial N or bacterial dry weight.

In the beginning (*MacFarlane,* 1907; *Brown,* 1913; 1914) standard and sample were compared with the naked eye, and the results were referred to the number of microorganisms measured by use of counting chambers. In 1953 the World Health Organization (*W.H.O.,* 1954) established an International Reference Preparation for Opacity. The aim was to enable workers in different laboratories to compare their results, and health authorities to formulate simple requirements for vaccines. A description of preparation and properties of the International Reference Preparation is given by *Maaløe* (1955). It consists of a suspension of small glass particles, roughly of bacterial size, in distilled water. The Preparation was made available by the American National Institute of Health, which used it since 1954 as a working standard for the characterization of pertussis vaccines and suspensions of **B.pertussis** used for challenge in the mouse protection test.

Although the International Reference Preparation is intended for visual

comparison, many investigators adapted the habit of using a photo-electric instrument. The instruments used for this purpose only give reasonable linear responses (according to the Lambert-Beer law) with molecular solutions. With suspensions deviations occur due to scattering of part of the light; the amount of scattering is dependent upon the optical construction and different from one instrument to an other. The method can only be used if one type of instrument is used, under carefully standardized conditions, using only the most linear part of the response curve. Fig. 3.37 shows the curve for the instrument used in this work. If suspensions are too concentrated they should first be diluted until they fall in the desired range of the instrument.

Difficulties have been encountered in standardizing later Reference Preparations against the original one. One is referred to the articles of *Spaun*

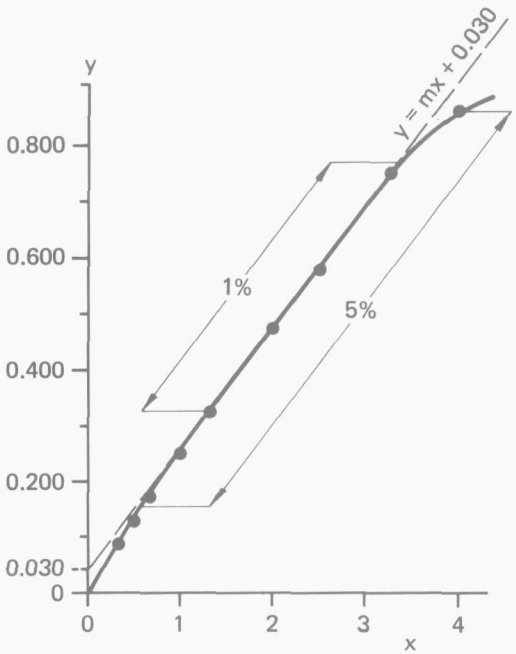


Fig. 3.37 Extinction as a function of concentration of a bacterial suspension.
Axis of abscissas (x): concentration of a suspension of **B.pertussis** in saline, expressed in arbitrary units.
Axis of ordinates (y): extinction, measured in a Vitatron-UC 200 SM photometer at 584 nm; tube diameter 10 mm; zero reference saline.
The percentages in the figure indicate the maximal deviation, within the given limits, from the line y = mx + 0.030, used for calculation.

(1962 a; 1962 b). In a recent study *Geser* (1970) concluded that there is at the moment a difference of 40 % between N.I.H. and W.H.O. Reference Preparations although they are supposed to be standardized against each other. A last argument that expression of bacterial cell concentration in opacity units is far from ideal is given in the study of *Benenson* and *Spaun* (1964) on the comparison of two typhoid vaccines. Eight laboratories using different photometers but the same Reference Preparation, found values from 3.0 to 7.6 for one of the vaccines.

It should be noted finally that standardization, even if one photometer in one laboratory is used, with reference to counted bacterial suspensions is very difficult. The optical methods measure bacterial mass rather than number of particles. So there is often a good correlation between optical density and bacterial dry weight, whereas later bacterial counts often reveal that the chosen ratio of numbers of cells to optical density has changed. This is mainly due to variation in bacterial cell size (see *Geser,* loc.cit.) or to change in the mean size of cell-aggregates, as e.g. with **M.tuberculosis** (see section 4.1.3). Fortunately the concentration of a vaccine is related, as practice has learned, better to bacterial mass than to number of cells. So measurement of optical density (or extinction) is still a practical and valuable criterion for determining bacterial concentration, provided it is not related to the actual cell count.

3.3.1. BATCH CULTURE

In batch culture the sterilized fluid culture medium is inoculated and the microorganisms are allowed to multiply. The desired product is gradually accumulated in the culture, either inside or outside of the cells.

The homogenization is effected by stirring with a turbine impeller. The vortex action of the impeller (cf. **Chain** et *al.,* 1952) guarantees the mixing with the gas atmosphere on top of the fluid. Normally a stream of air is directed towards the vortex. In special cases an other gas or a mixture of other gases is used, as e.g. oxygen and carbon dioxide in the production of staphylococcal α-toxin, or as nitrogen in the first stage of the production of tetanus toxin. It is selfexplanatory that, with oxygen control a mixed gas supply of varying composition has to be used.

Normally the culture conditions are followed in respect to temperature, pH and oxygen tension. The growth of the culture is estimated by determining the optical density (E), or the dry weight of the bacterial mass. In order to have a simple insight in the growth rate, and to facilitate

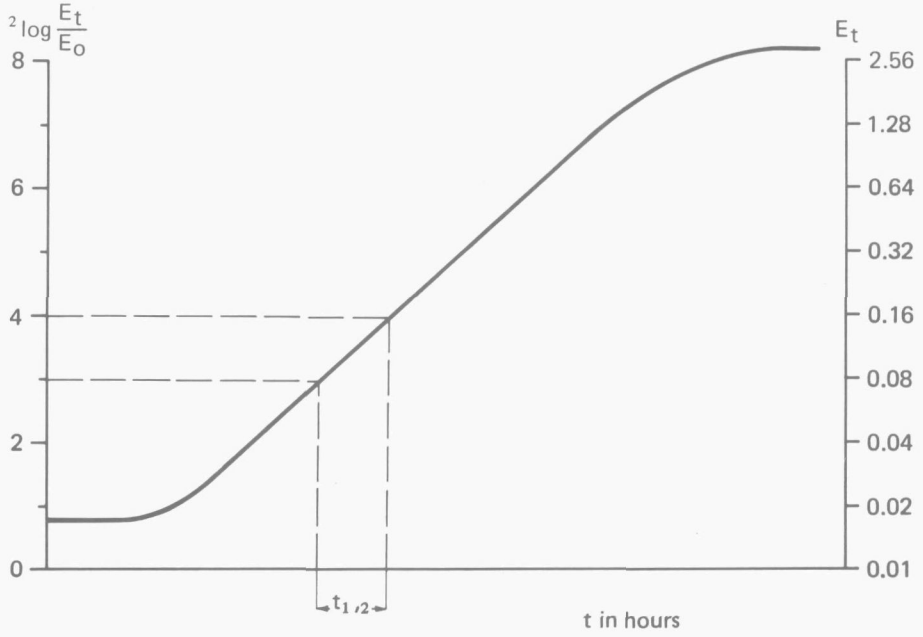


Fig. 3.38 Theoretical growth curve. On the right axis of ordinates the measured extinction at time t is given (E_t). E_o is not the extinction at time 0, but a fixed value of 0.01.
On the left axis of ordinates $^2\log \frac{E_t}{E_o}$ is given. Each unity means a doubling of E_t. The doubling time ($t_{1,2}$) may thus be easily calculated from the figure.

graphical analysis, the ratio $\frac{E_t}{E_o}$ is calculated, and from this value $^2\log \frac{E_t}{E_o}$. So an increase of $^2\log \frac{E_t}{E_o}$ with unity means doubling of the extinction. To be able, in addition, to compare several graphs with differing E_o, a fixed value has been given to E_o (normally 0.01). The principle applied to a graph where $^2\log \frac{E_t}{E_o}$ is plotted versus time is shown in Fig. 3.38. The application of this method of graphical representation means that the graph may start above or below the origin, but that every value on the axis of ordinates represents always the same extinction.

Although some of the processes to be described in Chapter 4 have a bacterial suspension as an end product, the product to be aimed at in vaccine production is always an antigen, or group of antigens. This is clear in the case of an extracellular product such as diphtheria toxin, but is also true in a case like pertussis, or cholera vaccine, where one is tempted to aim on cell yield:

but one should realize that a cell can contain, depending upon culture conditions, more or less antigen.

So, in every case where a method to estimate the amount of an antigen, correlated with immunizing potency is available, determination of cell yield is of secondary importance. It only comes into the picture where such a method is lacking. Often, in addition to the determination of the protective antigen, the presence of other factors, such as toxic factors has to be assessed. A good example will be given in section 4.1.1 (pertussis) where, besides the mouse protection test, determinations of at least two toxic factors are performed.

The application of kinetics, other than the empirical relationships between the variables mentioned, has been hampered by the fact that in vaccine production virtually never anything is known about the metabolic pathway by which the antigen is formed. Already many years ago, *Raynaud* et *al.* (1954; 1955) have pointed out that, in the case of exotoxins of different origin, the curves of bacterial growth and of toxin formation are not directly related. Antigens protective for man fall, in the designation of *Málek,* in the group of secondary products, i.e. products that are formed, not in direct conjunction with the metabolism connected with multiplication. Irrespective of whether this concept is of any value, it is clear that no simple kinetic considerations can be applied to the processes involved. The growth curve, although not essential in relation to antigen formation, is consulted frequently to evaluate the development of a culture. Besides the observation of physical criteria this is the most appropriate thing to do, and it has helped us considerably in establishing the primary requirement for any of the processes involved, namely a good growing culture.

In this respect three criteria have been of special interest:

1. duration of lag phase,
2. growth rate,
3. maximum cell density.

As to 1 (*duration of lag phase*) we have found that, with precautions it is almost always possible to avoid a slow start of the growth of the culture. In fact, it is not only the phase, in which the increase in cell material has not yet attained the logarithmic phase, (lag phase s e n s u s t r i c t o) but also the initial stationary phase which counts for us in this respect. A lag time (i.e. time from inoculation to onset of logarithmic growth) is considered appreciable by us if it exceeds 1 hour. In addition to inoculating medium pre-heated to cultivation temperature, we take several precautions with the inoculum. Whereas many investigators (and producers) use inocula in the order of 10 % of the final bacterial concentration, our experience

has shown that an inoculum of less than 1 % is often preferable. The inoculum should originate from a culture in the logarithmic phase, and be transferred immediately after withdrawal from the shaking machine. To achieve this, several shake flasks should be inoculated in series with an increasing amount of bacteria; after normal cultivation time (18-48 hours, depending upon organism and strain) at least one flask can be expected to have attained half maximal density and can therefore be considered to be in the logarithmic phase. It is possible that a culture, showing a considerable lag time due to an imperfect seed culture, subsequently grows normally and gives a good end product, but we have learned to distrust every culture with a longer lag phase than 1 hour; cf. Fig. 4.01 and p. 79.

In considering the *growth rate* (2) the following can be said. Theoretically if all nutrients (including oxygen) are in excess, growth may be called unlimited, unrestricted or balanced (a consideration of the several opinions on this point, which resulted e.g. in a very long discussion during the Continuous Culture Symposium in Porton in 1966, is beyond the scope of this thesis; we are, of course, aware of the possibility of limitation of growth due to so-called internal cellular factors). Under favorable conditions of, for instance, temperature and pH, a maximal growth rate is obtained. Even if the medium composition used is not optimal in all respects, a certain growth rate is obtained that is characteristic for the circumstances. A considerable deviation from this growth rate indicates a deviation from the conditions that had been found satisfactory for the process. This has occurred e.g. in pertussis vaccine production when an other batch of casein hydrolysate was introduced.

Although 3 (*maximal cell density*) has been designated as of secondary importance in relation to antigen production, it is still of value for the evaluation of the process; it finds therefore its place, along with the data of lag phase and growth rate, in the criteria to evaluate a culture. This is especially true in those cases (pertussis, typhoid) where no quick determination of the potency (or activity) of the product is possible.

In the processes studied considerable attention has been paid to the definition of the time at which the culture should be terminated (end point). The two criteria that are often used to determine this end point are end of growth or a certain predetermined culture time. Again, the solution is simple for products that are easily and quickly determined. For the products under consideration of which the formation is seldom parallel to growth, the first criterion is clearly not justified. The second criterion is even less applicable, because small differences in inoculum, lag time and growth rate result in considerable differences in the time to reach a fixed endpoint.

Therefore more valid criteria have been sought, especially for the processes with time consuming activity tests. In pertussis characteristic pH

Bibliotheek TU Delft

Magazijnslip/*Picking slip*

Ik wil dit document graag verlengen/*I would like to renew this loan.*

in te vullen door de Bibliotheek TU Delft/*to be completed bij the Delft University of Technology Library*

☐ verlengd tot/*renewed until :*

☐ niet verlengbaar, a.u.b. retour/*renewal not possible, please return the document*

☐ opmerkingen/*other* :

Briefkaart

FRANKEREN
ALS
BRIEFKAART

Aan de Bibliotheek van de
Technische Universiteit Delft
Postbus 98
2600 MG DELFT

Geleend op/*Date of issue*: Terugbezorgd op/*Returned on*:

14 36 26 08 1996

Uiterlijk terugbezorgen op/*Date due*:

23 09 1996 Paraaf/*Initials:*

Documentgegevens/*Received on loan*:

Hemert, P.A. van
VACCINE PRODUCTIONS AS A UNIT PROCESS

1154402 CBmg bkuitln 635018

Gebruikersgegevens/*Borrower*:

88546 Arkesteyn, W.M.----------

Dijkshoornseweg 49
2635 EK Den Hoorn

1932.306.6 nee

rise during cultivation has proved to be a good criterion in timing vaccine production. In B.C.G. cultivation attainment of the minimum oxygen tension has been useful. One is referred to Chapter 4 for the detailed description of these procedures.

Also, the reaching of a certain metabolic stage detected by level of carbon dioxide or oxygen in the gas phase might be a good criterion.

3.3.2 CONTINUOUS CULTURE

Continuous culture is defined here as the process in which, by continuous addition of medium and simultaneous withdrawal of culture fluid, a so-called "steady state" is established.

The theoretical basis for this specialized culture method has been laid by *Monod* (1950), and by *Novick* and *Szilard* (1950). A survey of their theory is given by *Van Hemert* (1962c); the main points are the following:

In the steady state, the dilution rate D (i.e. the replacement per hour, divided by the culture volume) becomes equal to the exponential growth rate μ (i.e. $\frac{1}{N}\frac{dN}{dt}$ where N is the bacterial density). Thus:

$$D = \mu \ (h^{-1})$$

From this the relation of dilution rate with the doubling time $t_{1,2}$ is easily deduced:

$$D = \frac{\ln 2}{t_{1,2}} = \frac{0.69}{t_{1,2}}$$

The mechanism is self regulating: if the bacterial concentration increases, the medium (or, more precisely the limiting factor) is further depleted, resulting in a decrease in growth rate. This results in a decrease in bacterial concentration, until the steady state is again reached. In the reverse case, the growth rate will increase reaching the steady state from the other side. The maximal dilution rate obtainable theoretically is the one at which the growth rate is maximal under the given circumstances. A further increase in dilution rate will cause wash out to occur.

The advantages of continuous culture have been advocated in the last decade by several groups of scientific workers, e.g. in Prague and in Porton. It is obvious that a higher yield can be obtained in the same culture volume in the same time. It is also obvious that optimal conditions for product formation can be arrested in time, if a true "steady state" has been reached.

Although the first consideration, production efficiency plays a secondary role for the vaccine requirements of a medium sized country, the latter

reason attracted our attention. Considerable work has been done on the production in continuous culture of pertussis vaccine as well as diphtheria toxin. In both cases a steady state could be maintained over a considerable time, namely 4-8 weeks. However, only in the cultivation of **C.diphtheriae** was a satisfactory production method realized.

There were two reasons for the failure in the case of **B.pertussis.**

1. During prolonged cultivation, some cells adhered to the sides and tubes of the culture vessel, forming clumps. Such clumps gradually turned dark. Microscopic observation led to the assumption that this phenomenon was due to partial lysis of the bacterial cells. Now and then these darkened clumps fell off into the culture and appeared in the effluent. The result was a cell suspension containing small dark masses. They could not easily be separated from the bulk, and lowered the quality of the product.
2. In some continuous cultures, the ability to produce protective antigen was lost after some weeks' cultivation. In view of the fact that the test results are known only after four weeks, this means that during that latter time an inferior product was being made.
   A phenotypic rather than a genotypic variation was most probably the reason for this phenomenon, because subcultivation of the abnormally behaving population (see Chapter 4 p. 95 and 96) produced the normal type of culture. It is easily understood, that in a continuous culture of this type the fastest growing part of the population will outgrow the slower. If the former part has a lesser antigen producing capacity, product formation will gradually decrease.

Where such a decrease is not occurring or where, in case it only seldom occurs, a quick determination method is available, the continuous culture method is applicable.

As will be shown in Chapter 4, a dependable continuous process method exists for diphtheria toxin production. According to the theoretical approach of e.g. *Málek,* a process in which growth and production curves do not coincide (as is the case here !) a continuous culture of at least two steps is required. Practice has not followed theory as far as diphtheria toxin production is concerned, and a one step process proved completely satisfactory.

For different reasons, however, we have not envisaged routine production of diphtheria toxin, using continuous culture. First, there is the quantity needed. In our case this is about 300 litres per annum. The higher yield in terms of toxin per litre culture volume per hour does not outweigh the more complicated preparations, necessary for a continuous culture, and the careful

observation required during the starting period.

For a steady state continuous culture further refinement of the technical set up is required. It is necessary to solve problems such as the constant addition of culture medium by pumping, the storage of medium supply and effluent, the sterilization of the large medium stocks or continuous medium sterilization, and the maintainance of a constant volume in the culture vessel.

Only in the case of diphtheria toxin production has it been possible to work out some kinetic aspects of the method, as will be described in section 4.2.1.

CHAPTER 4

Application of the Unit Process

The production processes for the vaccines prepared on a routine scale at the Rijks Instituut voor de Volksgezondheid, have been studied and developed according to the methodology described in the previous chapter.

The distinction between vaccines that consist of a cell suspension (particulate vaccines) and those that are formed by a product excreted by the cell (in this treatise only represented by detoxified toxins), is not fundamental from the point of view of production kinetics. This is explained as follows. Immunization is provoked, as has been indicated already on p. 68, by one or more antigens. Irrespective whether these antigens are excreted or remain an intrinsic part of the cell, as with particulate vaccines, culture conditions should always be directed towards maximal production of such antigen(s); with other words they are the guiding parameter in al processes to be described. Difficulties arise if no potency test is available. In the absence of a reliable potency test, cell yield is the only parameter left.

Excreted products, such as toxins, can generally be tested quicker and more accurately than the particulate vaccines. In the frame of this thesis the principles of potency tests will only be treated in detail where relevant. The same holds true for purification methods.

4.1 Particulate Vaccines

With the exception of B.C.G. vaccine, all particulate vaccines produced routinely in the Rijks Instituut voor de Volksgezondheid for immunization of man are in the form of a suspension of killed cells.

4.1.1 PERTUSSIS VACCINE

INTRODUCTION

Pertussis has been, and still is in countries with low health standards (*World Health Organization,* 1968) a disease that mainly attacks children. It is a highly infectious, airborne disease. After recovery from the disease a long lasting immunity ensues. The responsible microorganism, **Bordetella pertussis** has been discovered by *Bordet* and *Gengou* in 1906. For a good survey of the early history one is referred to *Nagel* (1970). The earliest successful attempt to prepare a vaccine was by *Madsen* (1933). No other indication for the efficacy of the vaccine, other than in a field trial, was available until *Kendrick* et *al.* (1947) presented the mouse protection test. In this test a group of mice is immunized with the vaccine and the survival after intracerebral infection with virulent pertussis bacteria is compared with that of a standard vaccine. Field trials performed in England by the *Medical Research Council* (1956; 1959) proved the correlation between the mouse protective value (in IU per human dose) and the immunogenic power in children. The test takes three to four weeks and gives, even with the best precautions and using 60 mice per test, insufficiently accurate results, with 95 % confidence limits of 0.4-2.5 of the calculated mean value.

Besides the assessment of potency in the mouse protection test, the W.H.O. requirements prescribe a toxicity test commonly called the mouse weight gain test. In this test the weight gain and eventual death is observed after seven days. A normal suspension of pertussis cells contains a thermolabile toxin that is inactivated by the standard procedure of heating at 56°C for 10 minutes. Besides being a check on this inactivation procedure, the test has also proven useful in the detection of a thermostable toxin, mainly present in the supernatant (cf. p. 87).

Sensitization to histamine, which develops after injection of mice with pertussis vaccine, has been ascribed to a distinct factor called histamine

sensitizing factor (H.S.F.). An estimation of the amount of this factor can be made in mice. The results of this determination often run in parallel with mouse protective activity, as is shown in several tables given on the next pages. The factor has been the subject for speculation by many workers: a scale of possibilities between complete identity of it with the protective antigen and virtual independence of the two have been advocated. *Nagel* in his thesis (1970) gives a method to separate the protective power from H.S.F. Although this does not prove complete independence, the method is worth to be considered for obtaining a H.S.F.-free vaccine (cf. also p. 100). This is important because H.S.F. is considered by some workers responsible for serious complications following pertussis vaccination (*Sanyal,* 1961).

The oldest method for cultivation of **B.pertussis** was on *Bordet-Gengou* agar (containing sheep blood) in Petri-dishes or Roux-flasks. It is clear that the method did not lend itself to mass production. *Hornibrook* (1939; 1940) was the first to use a fluid medium. Further developments came from *Cohen* and *Wheeler* (1946) and *Verwey* et *al.* (1945; 1949). Also the work of *Rowatt* (1957 a; 1957 b) has contributed to our knowledge; she studied among others the role of inhibitors present in the medium of *Cohen* and *Wheeler. Proom* (1955) showed that inhibition of growth of **B.pertussis,** when grown in a medium with cysteine, is caused by decomposition of this compound into colloidal sulphur and sulphides through autoclaving. The inhibition is overcome when cysteine, sterilized by filtration, is added separately (*Woiwod,* 1954). Inhibition by unsaturated fatty acids is most probably counteracted by the presence of starch or charcoal. *Rowatt* (loc.cit.) postulated the presence of a third inhibitor, which is counteracted by blood; hence her conviction that no rapid and profuse growth will be obtained without blood, added deliberately, or adhering to the inoculum, which has been taken from a medium containing blood. We have not been able to confirm this last finding, and have obtained satisfactory growth and yield with inocula from media without blood.

The most recent impetus to the knowledge of the growth requirements has been given by *Stainer,* as will be discussed in a separate section.

DEVELOPMENT OF CULTIVATION METHOD

The first culture medium used was the medium of *Cohen* and *Wheeler* (1946), with the Lister Institute modification (*Standfast,* pers.comm.). The main constituents of this medium are an acid casein hydrolysate, cysteine, glutamic acid, yeast extract and salts.

A further adaptation of this culture medium to our conditions was performed on the following grounds (see Table 4-01 for a comparison between the Lister medium (L-1) and the B-2 medium, developed by us):

Table 4-01 Composition of L-1 and B-2 medium in g/l.

	L-1	B-2
Casein hydrolysate, Oxoid	2	-
Casamino acids, Difco techn.	-	2 + 4*
l. glutamic acid	5	5
NaCl	2.5	2.5
KH_2PO_4	0.05	0.5
$MgSO_4 . 7$ aq.	-	0.1
$MgCl_2 . 6$ aq.	0.4	-
$CaCl_2 . 6$ aq.	0.01	0.01
$FeSO_4 . 7$ aq.	0.0125	0.0125
$CuSO_4 . 0$ aq.	0.0005	0.0005
d.l. cysteine HCl	0.03	-
glutathion	-	0.01
yeast extract (freshly prepared)** (ml)	5	50
starch	1.5	1.5

pH = 7.2

* autoclaved separately

** Yeast extract is prepared as follows:
Baker's yeast 1000 g
Aqua dest. to 5000 ml
Adjust pH to 7.6 with 10 % NaOH. Autoclave 30 minutes at 108°C. Filter through "hot agar paper", after mixing with 40 g diatomaceous earth per litre volume.

1. The Oxoid casein hydrolysate had to be replaced by Difco technical casamino acids, because of great batch differences with the former. We found that part of the Difco technical casamino acids had to be added after the sterilization of the bulk of the medium to obtain optimal results.
2. Freshly prepared liquid yeast extract gave the best results, provided the original amount was increased. Nicotinic acid could replace yeast extract, but this alternative was rejected, because a lag was sometimes observed.
3. Cysteine was replaced by glutathione. It appears that the microorganism can accept the cysteine in this form and this tripeptide, unlike cysteine, is not decomposed by sterilization.
4. In the L-1 medium the Mg-concentration is higher than in several comparable compositions (*Verwey* et *al.*, 1949; *Jebb* and *Tomlinson*, 1955). The Mg-concentration was reduced when we found that the

higher concentration had no advantage and, moreover, gave rise to a deposit of $MgNH_4PO_4$, especially in the effluent line during continuous cultivation.

Several data on experiments, given below, have already been published by us earlier (*Van Hemert,* 1964d; 1969).

A typical growth curve is shown in Fig. 4.04. There is almost no lag phase, provided the inoculum is in the logarithmic growth phase. A comparison of two cultures seeded with inocula of different age is given in Fig. 4.01. With the younger seed culture (characterized by lower cell density and lower pH), which is assumed to be taken in the logarithmic growth phase, there is no detectable lag phase, which is in contrast with the other culture, seeded with an appreciably older inoculum. The doubling time normally obtained is three to four hours, in accordance with the data of *Rowatt* (1957 b). She also mentions a higher growth rate, with a doubling time of about 2.5 hours at low cell densities. The existence of two distinct growth rates is also shown in

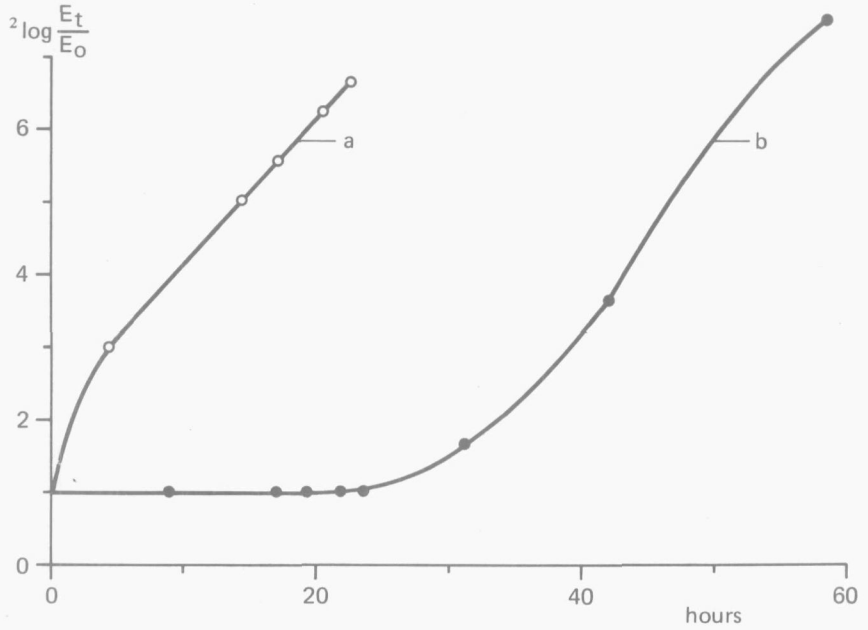


Fig. 4.01 Influence of inoculum on lag phase.
B.pertussis, strain 134.
a. inoculum Ext. = 1.15; pH = 7.7.
b. inoculum Ext. = 2.20; pH = 8.0.

our results, see Fig. 4.01 and 4.04. Cell yields of 60-80 x 10^9 germs per ml are routinely obtained. The yield of protective antigen, as determined with the highly inaccurate mouse protection test is normally above the W.H.O. limit of 4 IU per human dose, as is shown for a typical production series in Table 4-02 (a human dose contains invariably, in the cases described, 16 x 10^9 cells, as determined by opacimetry).

The pH goes up during the cultivation process from 7.0 at the beginning to beyond 8.0 at the end of growth, rising further during prolonged incubation. It is known that with increasing pH-values the protective antigen becomes less stable. To determine the optimal point for the end of the culture, the protective value (per cell and per ml) has been studied as a function of pH during cultivation; cf. Fig. 4.02 and 4.03. Fig. 4.02, recording the protective activity per ml of culture volume, gives an impression at which pH the antigen yield is maximal. Although no line has been drawn through the points, a pH around 8.5 may be considered as optimal in this respect. Because a human dose contains a fixed level of cells (16 x 10^9 cells), the value per human dose, as given in Fig. 4.03 is, however,

Table 4-02 Cell yield and mouse protective activity of a typical production series of *pertussis vaccine* (May '69 to January '70). 40 litre culture in B-2 medium.

Batch no.	Strain	Yield, litres of 128 x 10^9 cells/ml	m.p.a. IU/human dose
297	509	11.8	30.6
300	134	17.5	6.4
301	134	17.8	7.2
303	134	16.7	14.0
307	509	15.0	10.4
308	509	14.5	6.8
309	134	16.2	6.8
311	509	10.5	7.8
312	134	15.2	7.8
315	509	11.7	11.2
316	134	14.2	7.6
327	509	15.0	8.8
329	509	19.1	17.2
330	134	19.2	8.8
331	509	18.8	10.2
332	134	18.8	10.6

N.B. Interlying batch numbers have mostly been used for experimental purposes.

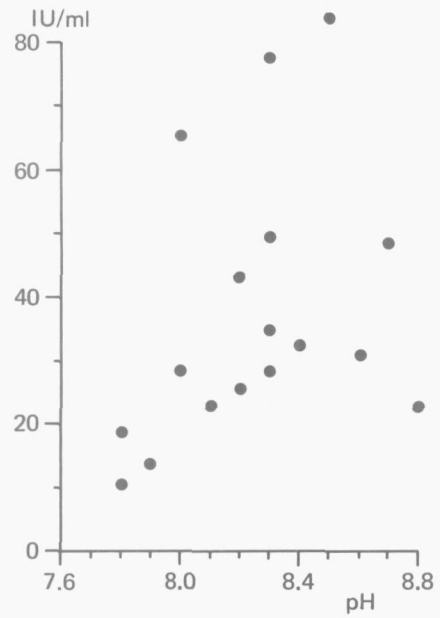


Fig. 4.02 Relation between pH and mouse protective activity per ml in samples from 6 regular cultures of **B.pertussis** in B-2 medium.

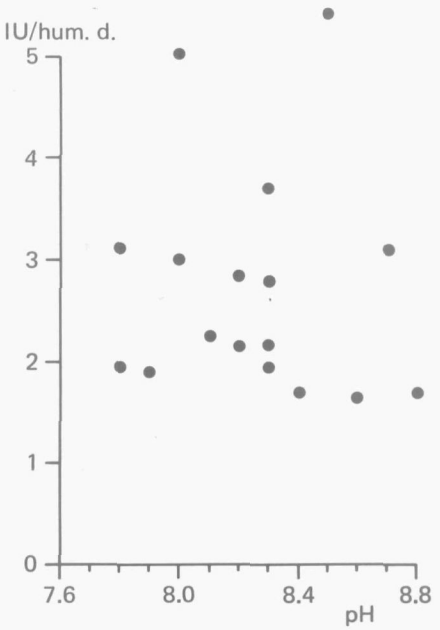


Fig. 4.03 Relation between pH and mouse protective activity per human dose in samples from 6 regular cultures of **B.pertussis** in B-2 medium.

of greatest importance. This figure shows that cultivation can be prolonged until a pH of at least 8.4 is reached.

Several other experiments have been performed with pH-control. No advantage in terms of cell yield has been found, whether the pH was held constant at 6.8, 7.2 or 7.8. In some of the pH-controlled cultures there was an unexpectedly high protective power after prolonged cultivation. Although we are convinced of the reality of this phenomenon (Table 4-03), it proved to be rather ephemeral: repeated tests after a few months showed that the values decreased to normal ones. The fact could be explained by postulating the presence of two forms of protective antigen, one being relatively unstable, or alternatively the existence of an adjuvant that is equally unstable and only formed under the special conditions of the experiment.

In normal cultures the pO_2 gradually decreases, reaching negligible values only during the later stages of growth, as a typical curve shows (Fig. 4.04). Oxygen control at a pO_2 of 100 mm Hg retards growth, whereas control at

Table 4-03 Stability in respect to mouse protective activity (in IU per human dose) of samples, taken 24 hours after maximal density, from cultures with pH-control at 7.2 (upper half). In the lower half of the table results with samples, taken earlier from the same cultures, are given.

batch and sample	m.p.a. IU/hum.d.		x
	direct	after x months	
236-IV	24.0	10.0	4
258-VII	37.8	6.8	3
261-XI	44.4	8.4	2
236-I	7.8	13.6	4
258-IV	9.0	8.2	3
261-IV	4.2	1.2	2

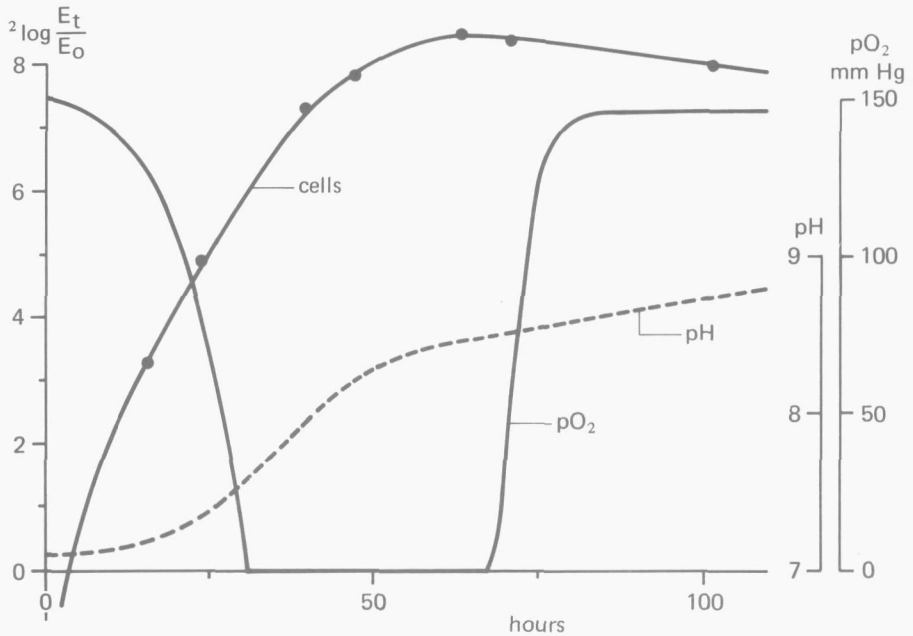


Fig. 4.04 The course of cell density, pH and pO_2 in a normal culture of **B.pertussis**, strain 134 in B-2 medium.
Culture volume 7 litre. Air flow rate 1.5 l/min.
Stirrer speed 450 r.p.m.

22 mm Hg has no appreciable influence on growth rate, compared with a non-controlled culture. Comparison of the consumption of several amino--acids with control at 22 mm Hg and without (pO_2 certainly lower during part of the process) indicated that they are consumed faster at a higher pO_2 (cf. Fig. 4.13 and 4.14 and p. 99). Our results of determinations of the amino-acid consumption are in good accordance with those of *Lane* (1970) that became known to us after the completion of our experiments. No pO_2-control was studied by *Lane*.

Thus it appears that amino-acids are dissimilated quicker at higher pO_2-values. This is supported by the following finding: normally carbon dioxide evolution comes to an end soon after maximal growth has been reached. Addition of glutamic acid after this point gives rise to rapid evolution of an amount of carbon dioxide equivalent to total dissimilation (5 M CO_2 from 1 M glutamic acid).

At very low pO_2-levels there will be less dissimilation, and the amino-acids will remain available for other biochemical processes for a greater part of the cultivation. If some amino-acids were to play an essential role in the formation of the protective antigen, cultivation near a pO_2 of zero may be important. Further experiments are in progress now, using, among others, continuous cultivation, to observe the relationship between the pO_2-level and concentration of the most important amino-acids during cultivation, and the subsequent effect on the mouse protective activity.

ACID PRECIPITATION

Cells are normally harvested after growth by centrifugation. Several types of commercially available continuous flow centrifuges have been tried. Some did not ensure aseptic working, while in others the cells were heated excessively. The most difficult step was the collection of the sediment from the rotor. Thus continuous centrifugation was not applied to the harvest of particulate vaccines.

A satisfactory routine process for batch centrifugation was developed as follows:

The fermentor is disconnected from the panel and transferred to a room for aseptic work. With a little overpressure the culture fluid is dispensed in 1 litre transfusion bottles, capped, and centrifuged for one hour at 1300 g. The method of decantation, addition of saline, resuspension and transfer to a B_{20} bottle (cf. p. 30) is shown in Fig. 4.05.

Although the method is fairly safe for the laboratory worker and contamination is on average less than 5 %, it can still be considered as a limiting technical factor in vaccine production especially as it does not lend itself to scaling-up.

It is therefore that the harvesting by acid precipiation, developed by

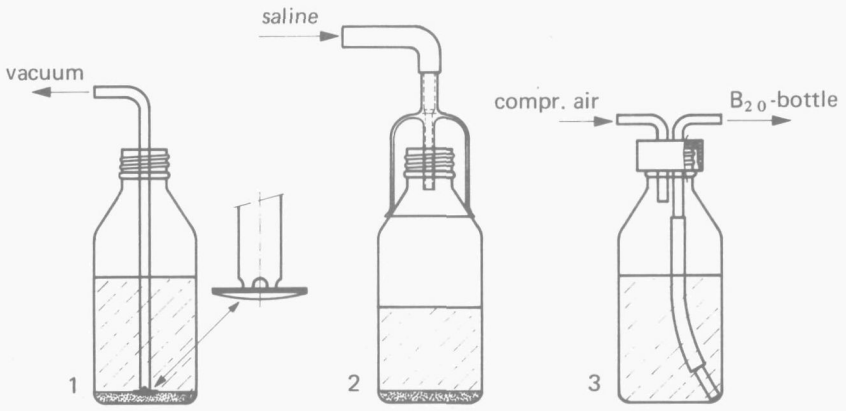


Fig. 4.05 Schematic representation of normal processing of **B.pertussis** suspension.
1. (After centrifugation) removing the supernatant.
2. Addition of saline before resuspension.
3. Transfer of suspension to pool.

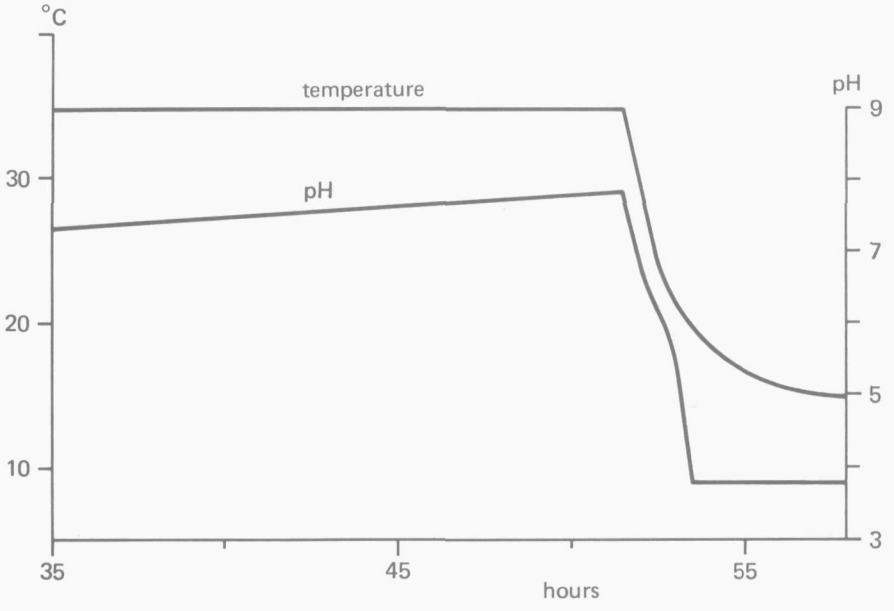


Fig. 4.06 The course of temperature and pH in a culture of **B.pertussis** with automation.

Pusztai, Joó and *Juhász* (1961) was considered as a progress. The method consists of the acidification of the grown culture by hydrochloric acid to pH 4.0. The cells quickly sediment, and can be collected in 3-5 % of the culture volume. The method lends itself very well for automation, e.g. in the following way. On reaching the culture endpoint (pH 7.8-8.4) the setpoint of the pH-controller is actuated and starts HCl addition while heating is switched off. At pH 4.0 the other pH setpoint stops the acid pump and the stirrer. The culture is then allowed to settle. Fig. 4.06 gives the course of temperature and pH in such an automated culture process.

After centrifugation and resuspension in saline, a vaccine is formed with the same average mouse protecting activity as comparable centrifuged vaccines. The method is applied quite extensively elsewhere, but has not been accepted in the R.I.V. for reasons stated below. In Table 4-04 the results of the mouse protection test are given for five cultures. One half of the culture was processed by centrifugation, the other half by acid precipitation. Even while taking into account the wide confidence limits inherent to this test (cf. p. 76), the centrifuged samples are seen to show somewhat higher activities.

All vaccine batches prepared by us, using acid precipitation, passed the N.I.H. mouse weight gain test; in this test a limit of 3 g is set irrespective of the weight of the control mice. *Van Ramshorst* (1969) suggested a modification of the evaluation of the test, only accepting vaccines giving a weight gain of at least 60 % of the control (see also *Van Hemert,* 1967).

Table 4-05 gives a comparison of vaccines prepared according to the two methods. The acid precipitated vaccines consistently show a higher toxicity, although they are all above the 60 % limit of *Van Ramshorst* (loc.cit.). This

Table 4-04 Comparison of mouse protective activity in centrifuged with that in acid-precipitated part of five cultures.

Exp. no.	strain	m.p.a. IU/human dose	
		centrifuged	acid-precipitated
263	509	12.2	8.4
267	134	8.2	5.6
292	134	12.6	9.0
294	509	10.8	2.2
295	509	14.4	7.4

Table 4-05 Comparison of results of weight gain test of centrifuged, compared with acid-precipitated pertussis vaccines. Given are the mean values per group of the percentual weight gain (control group = 100 %).

Treatment	Number of Batches	Weight Gain
Acid-precipitation	14	69
Centrifugation	16	102

Table 4-06 Percentual weight-gain of mice, injected with DTP-polio vaccines (control group = 100 %).

Batch	Treatment Pertussis	% weight gain
40	centrifugation	100
41	centrifugation	102
42	centrifugation	63
43	acid-precipitation	28
44	acid-precipitation	42
45	centrifugation	71
46	centrifugation	107
47	centrifugation	84
48	centrifugation	107
49	centrifugation	69
50	centrifugation	65
51	centrifugation	75
52	centrifugation	73
53	centrifugation	93
54	centrifugation	70

is even more pronounced, if DPT-polio vaccines, containing besides pertussis vaccine, polio vaccine and diphtheria and tetanus toxoids are compared as is shown in Table 4-06 (see also *Van Hemert,* 1969).

The supposed correlation between the N.I.H. test and the toxicity after injection in human beings, has never been proven. The acid precipitation method was, however, abandoned, because the indication of a higher toxicity could not be neglected. But research was started on the phenomenon. In a sequential analysis of the results after the injection of children with four DPT-polio batches, two with centrifuged and two with

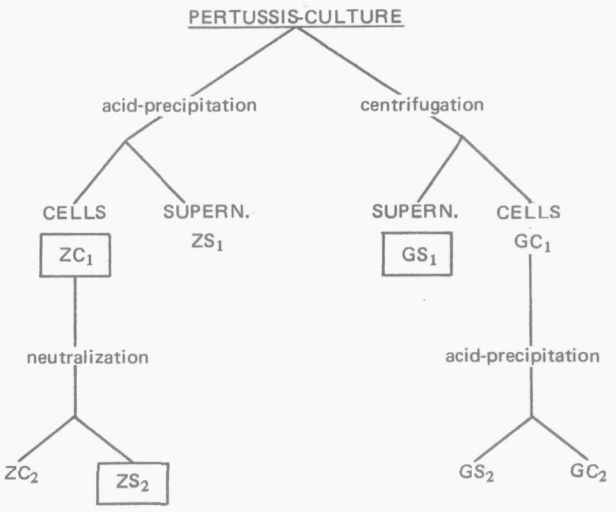


Fig. 4.07 Schematic representation of an experiment in order to demonstrate the precipitation of the supposed toxic factor at low pH.

acid precipitated pertussis vaccine, *Cohen* (1968) observed a slight difference between the two pairs in favour of the former. Although the effect was much smaller than in the weight gain test, the validity of this test as an indication of acceptability of pertussis vaccine was stressed.

During laboratory investigations on the toxic behaviour of acid precipitated pertussis vaccine we found that performance of the mouse toxicity test sometimes gave more information when an eight times higher dose was injected. In particular the percentage of dead mice after three and seven days proved to be informative. With this extended toxicity test as a tool we found a series of properties which, to our belief, have to be ascribed to a distinct toxic factor.

We found that the postulated factor precipitates at low pH (Fig. 4.07). On centrifuging a pertussis culture, the factor remains in the supernatant (GS_1), whereas during acid precipitation it coprecipitates with the cells. However, it redissolves after neutralization, and is thus found in the supernatant (ZS_2), when the neutralized cell suspension is centrifuged. The culture medium itself proved to be non-toxic before inoculation.

Although all samples, pertaining to this scheme, were heated for 10 minutes at 56°C, the normal detoxification conditions for pertussis suspensions, it was seen that extra heating of the S_2-fractions gave no appreciable decrease of toxicity. The toxic factor is therefore of a thermostable nature.

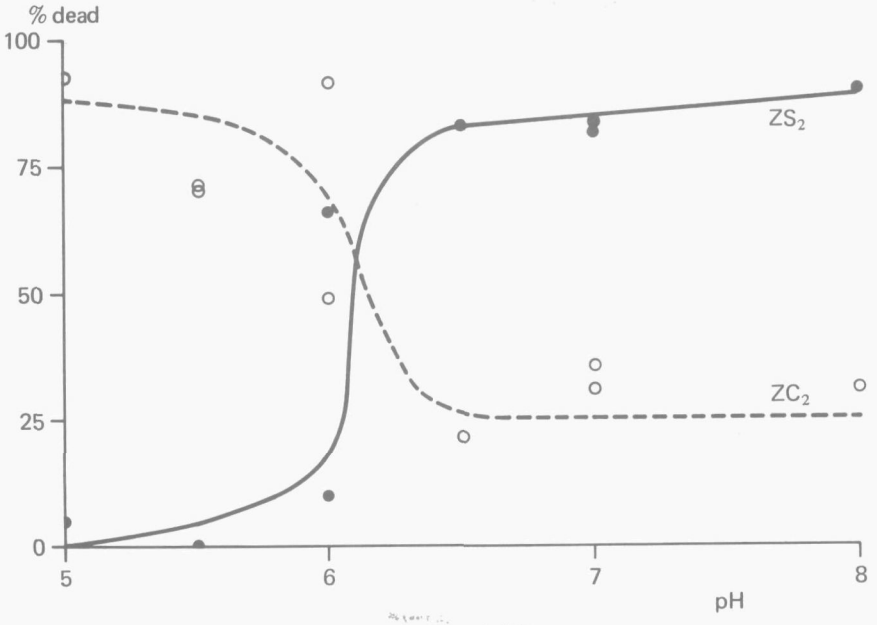


Fig. 4.08 Division of thermostable toxin of **B.pertussis** over ZS_2- and ZC_2-fractions at different pH-values. Percentage dead mice in toxicity test at eightfold level.

Table 4-07 Purification of acid-precipitated pertussis vaccine. Comparison with centrifuged vaccine (lower part); C_1 = untreated acid-precipitated or centrifuged cells; C_2 = treated cells.

Batch	% dead at 8 fold dosis		% weight gain at normal dosis	
	C_1	C_2	C_1	C_2
104	95	25	63	91
107	85	55	46	78
110	90	15	37	76
113	75	95	47	66
114	100	45	36	80
120	90	75	−27	83
108	0	-	68	-
112	5	-	81	-
115	10	-	98	-
118	0	-	91	-

Table 4-08 Comparison of two dutch and three hungarian strains of **B.pertussis.** Figures are mean values of two, in strain 134 of four experiments.
% weight gain: 0.5 ml of 16 x 10^9 cells/ml with mice after 7 days; control = 100 %.
% dead: 0.5 ml of 128 x 10^9 cells/ml with mice after 7 days.
m.p.a.: mouse protective activity in IU per human dose.

strain	centrifuged			acid-precipitated		
	% w.g.	% dead	m.p.a.	% w.g.	% dead	m.p.a.
134	89	30	5.8	52	93	7.4
509	101	5	7.0	39	98	8.8
358 E	78	73	7.8	64	98	6.6
2894	96	43	7.6	60	98	10.0
41405	103	13	7.8	79	95	6.0

In Fig. 4.08 the toxicity of cells and supernatant of a suspension of acid precipitated cells, in relation to pH, is given. Here the percentage death in mice, seven days after injection with an eightfold dose, is used as a criterion. A gradual shift of the toxic factor from the cells to the supernatant between pH 5 and 7 is observed. Therefore, and taking into account that the protective value is most probably damaged above pH 7.5, pH 6.8 was chosen for treating the acid precipitated suspension.

Although it is possible to diminish in this way the "extra" toxicity of acid precipitated cells, they always remain more toxic than comparable centrifuged cells (Table 4-07). For this reason and because the method had already lost part of its attractivity by the necessary extraction, it has been abandoned.

Speculations about the nature of the thermostable toxic factor have been made. Purification experiments have been performed, giving a product of a lipopolysaccharide nature. It resembles the "late appearing toxin", described by *Kurokawa* et *al.* (1965), in respect to the delay with which mice are dying (between the third and seventh day) at the eightfold dose in the supernatant.

In addition, a comparison has been made between the two strains, normally incorporated in the Dutch vaccine on the one hand and three strains that have been received from *Dr.I.Joó* (Institute "Human", Budapest) on the other hand. The results shown in Table 4-08 indicate that percentual weight gain of acid precipitated vaccines is always lower than of centrifuged

ones. Although the Hungarian strains are a little better in this respect than the Dutch ones, the lethality at the eightfold dose level clearly indicates that for all strains, centrifugation leads to a better vaccine. The reason that, as has been mentioned already, the method of acid precipitation is in use in several laboratories for vaccine production, lies perhaps in the fact that, in most cases, the end product will pass the official N.I.H. toxicity test. Evidently it is desregarded that better results are obtained with centrifugation, resulting, as the work of *Cohen* (loc.cit., 1968) shows, in a vaccine less toxic for children.

CONTINUOUS CULTURE

The experiments with continuous culture of **B.pertussis** (*Van Hemert,* 1962a; 1964a; 1964d; *Cohen* and *Van Hemert,* 1963) are of note, although no final success has been obtained with this method for routine production of pertussis vaccine, as has been mentioned already on p. 72. The findings have indicated how to proceed to obtain a good "steady state". It is conceivable that under other circumstances the method is applicable, e.g. if a multiple of the present quantity is needed for purification purposes.

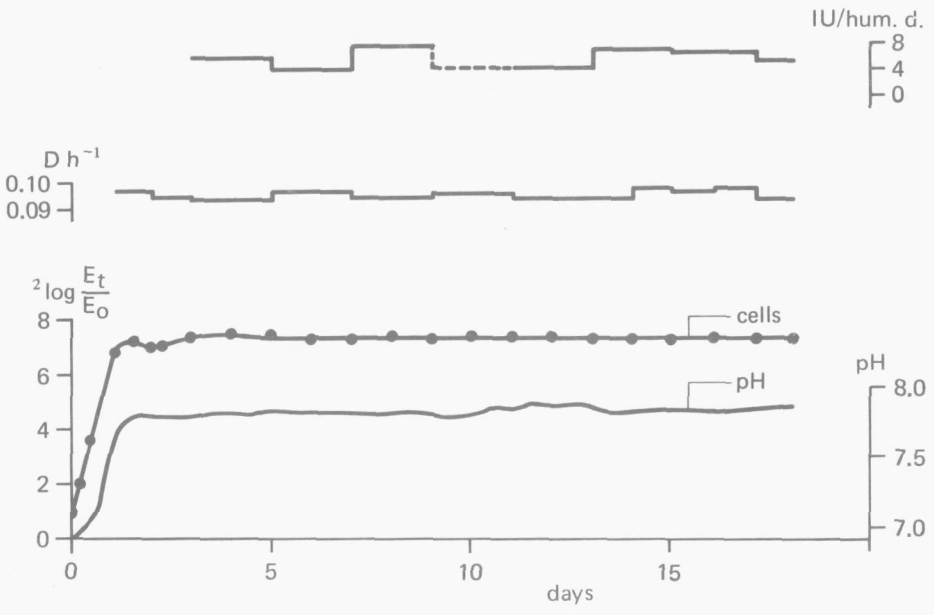


Fig. 4.09 Continuous culture of **B.pertussis,** strain 509.
Mouse protective activity in IU per human dose.

Table 4-09 Mouse protective activity in parallel continuous cultures of **B.pertussis,** strain 509. Flow rate D = 0.09 - 0.10 h^{-1}.

Parallel cultures	sample after (days)	IU/human dose	
		first culture	second culture
77	4	12.4	12.0
and	8	10.4	5.4
78	12	10.2	4.0
	4	8.6	5.2
80	12	4.8	14.8
and	16	3.4	5.2
81	20	5.0	2.2
	28	6.6	1.8
	30	9.4	15.6
89	4	6.6	9.6
and	8	22.0	23.6
90	18	2.2	6.8
	20	7.4	13.4

The initial problems were of technical nature. Experience had to be gained with pumping systems for accurate continuous flow. The Sigmamotor peristaltic pumps proved to be completely adequate, provided the regular speed variator (Zeromax) had been replaced by a more dependable type (Contraves). The clogging of the effluent line by $MgNH_4PO_4$-crystals was prevented by changing the Mg-concentration in the medium, as mentioned (p. 78). Contamination problems were successfully overcome when C-containers were installed for medium storage; medium can be filtered into, or can be sterilized in the container itself. A B-20 container was permanently attached to the effluent. When filled, the container is emptied by overpressure by a tube reaching to the bottom, leaving most of the darkened clumps in the foamy top layer.

The first series of continuous cultures was performed with a dilution rate (D) of about 0.10 h^{-1} (Fig. 4.09). Table 4-09 gives a record of three cultures in duplicate; two cultures are run in parallel with the same batch of medium to nullify medium batch differences. Taking into account the inaccuracy of

the mouse protection test, there is no evidence of a gradual decrease in protective power in the course of cultivation.

A gradual increase in cell concentration in the first days of continuous flow is regularly observed. This is in accordance with the phenomena shown in Fig. 4.10 and 4.11. In Fig. 4.10 three growth curves are shown, the first directly derived from the seed culture of a continuous culture, the second from a sample after five days, the third from a sample after 24 days. It is clear, especially when the third curve is compared with the first one that a higher growth rate is maintained until higher cell densities. It is also shown in the figure that the growth rate corresponding with a $D = 0.095\ h^{-1}$, i.e. a doubling time of 7.3 hours, is reached in the second and third curve at a cell density that is nearly the same as the steady state cell density at the moment of sampling. In Fig. 4.11 continuous flow is discontinued for 12 hours; after

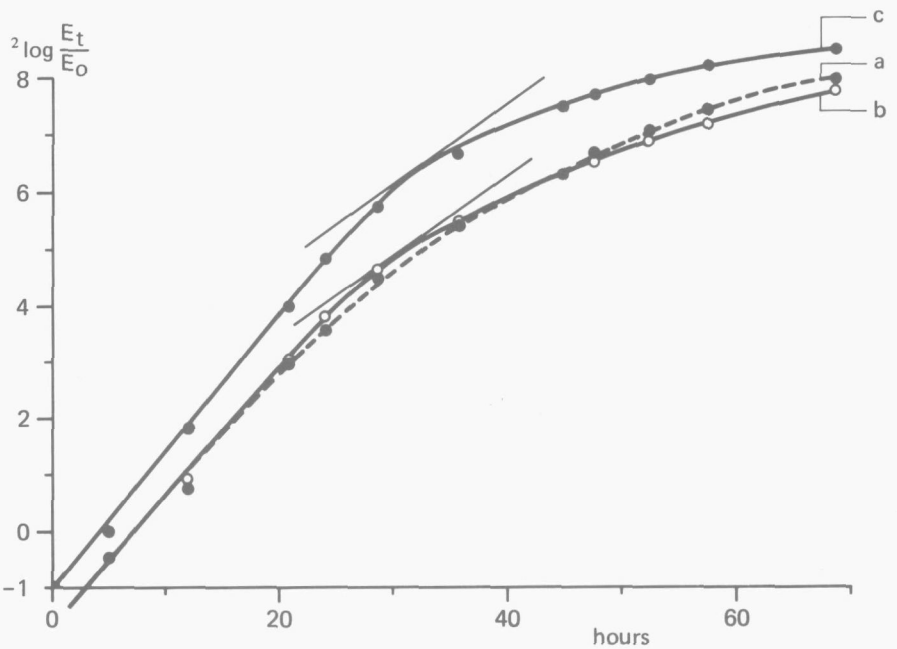


Fig. 4.10 Comparison of growth curves of batch cultures derived from different stages of a continuous culture of **B.pertussis,** strain 509.
a. seed culture,
b. after 124 hours,
c. after 576 hours.
Flow rate of original culture $D = 0.095\ h^{-1}$.
The slope of the tangents corresponds with a doubling time of 7.3 hours.

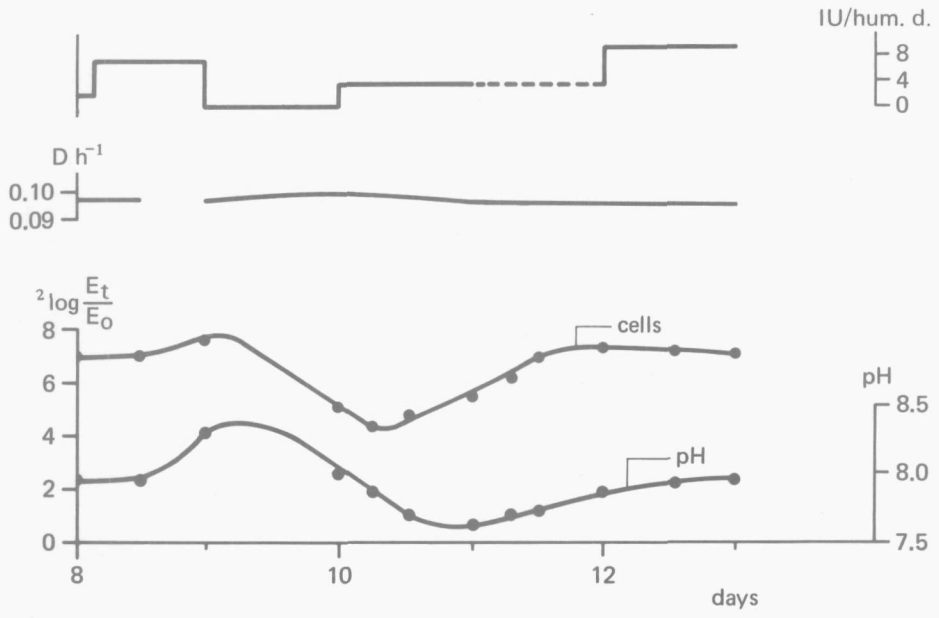


Fig. 4.11 Continuous culture of **B.pertussis,** strain 134.
Effect of interruption of continuous flow on cell density, pH and mouse protective activity.

this the onset of continuous flow almost caused a "wash out" of the culture, indicating that the population had changed its behaviour. The imbalance lasted several days before the original equilibrium was regained. Microscopically a difference was observed: long filamentous cells were formed after the discontinuation of the continuous flow, but the regular coccoid forms were almost exclusively present, once equilibrium was reached.

In continuous cultures in which, through inaccuracy of the pumping system, a small variation of dilution rate and hence of the cell density occurred, the pH shifted up and down with the cell density. So observation of the pH-recording provided an easy check on the constancy of the dilution rate.

The influence of dilution rate on cell density and protective activity was observed by changing the dilution rate in one culture and assuming equilibrium after a few days. However, complete equilibration could not be expected in that time. The only dependable way is to start comparable cultures with different constant dilution rates. Table 4-10 gives such a comparison. No difference in mouse protective activity per cell could be

Table 4-10 Continuous culture of **B.pertussis,** strain 509. Results at different dilution rates.
Medium B-2; mouse protective activity and histamine sensitizing factor in units per human dose (16×10^9 cells) in centrifuged samples.

Exp.	D in h^{-1}	Sample from day	till day	M.P.A.	H.S.F.
129	0.060 ± 0.001	2	5	2.4	-
		5	9	7.8	2.2
		9	13	5.8	1.8
		13	16	5.0	< 0.2
136	0.080 ± 0.001	4	7	22.0	-
		7	9	29.2	-
		12	15	38.6	3.2
		15	18	7.0	4.8
		18	(outgrowth)	13.8	12.2
145	0.040 ± 0.001	1	6	7.2	7.4
		6	11	7.0	11.6
		11	15	5.4	13.4
		15	18	10.4	7.4
		18	21	3.6	12.4

ascertained. Maximal cell yield (per culture volume per unit time), and thus probably maximal yield of protective activity, was obtained at a $D = 0.06\ h^{-1}$.

The results obtained in a series of continuous cultures at a $D = 0.06\ h^{-1}$ are shown in Table 4-11. With the possible exception of experiment 160, no decrease in protective activity is detectable.

The cells from the continuous culture of **B.pertussis** have been used repeatedly as a starting material for solubilization experiments (*Van Hemert, Van Wezel* and *Cohen,* 1964; cf. *Nagel,* 1970; *Van Wezel* and *Van Hemert,* 1967b). This solubilization was performed with sodium deoxycholate. Addition of DNA-ase was necessary to reduce the viscosity of the solubilizate before further treatment. When cells from a culture with a $D = 0.06\ h^{-1}$ were used instead of those from a culture with a $D = 0.10\ h^{-1}$ RNA-ase was also needed. This was a rather unexpected phenomenon; *Herbert* (1959) mentioned an increase of the RNA-content of the cell with increasing D for **Aerobacter aerogenes** and **Staphylococcus aureus;** *Sykes* (1966) gave a rather

general statement to the same effect. The observation has not been the subject of further investigation.

In some extended continuous cultures a significant decrease in protective power per cell accompanied by a changing behaviour of the cells themselves has been observed. Experiment 144 (Fig. 4.12) is taken as an example. There is a gradual decrease in mouse protective activity; on the 37th day long irregular cells appear, and on the 39th day, the culture could no longer be precipitated by acidification. There was no contamination. The culture has been lyophilized and rechecked six years later. It has been sent, moreover to *Dr.M.A.Aprile* (Toronto), studying the transition of **B.pertussis** from phase I to phase IV.

This transition was first described by *Leslie* and *Gardner* (1931). Fresh isolates from patients are in phase I; subculturing sometimes leads to changes in antigenic behaviour, considered as a gradual transition from the smooth to the rough form, and designated as phase II, III and IV. *Gray* (1946) showed that these phases are less immunogenic than phase I. Our own findings made a true phase IV behaviour improbable, because after subcultivation the cells returned to normal, coccoid forms, and could again be precipitated by acidification.

Table 4-11 Continuous cultures of **B.pertussis.**
Strain 509; medium B-2; D = 0.06 h^{-1};
mouse protective activity and histamine sensitizing factor in units per human dose (16 x 10^9 cells) in centrifuged samples, taken at a certain time interval (from day... to day...).

Exp.	day/day		M.P.A.	H.S.F.	Exp.	day/day		M.P.A.	H.S.F.
129	2	5	2.4	-	148	1	9	(1.8)	9.6
	5	9	7.8	2.2		9	16	12.2	5.2
	9	13	5.8	1.8		16	23	12.8	8.6
	13	16	5.0	<0.2					
					160	2	4	25.2	2.8
154	2	3	7.3	-		10	12	2.4	4.8
	8	9	6.0	1.0					
	14	16	6.4	-	164	2	4	5.6	9.0
	18	20	3.6	-		12	14	9.0	6.2
	25	27	6.4	6.4		17	19	13.6	-
	33	35	8.6	5.4		25	27	12.8	-
						31	33	21.0	-
						39	41	22.0	-

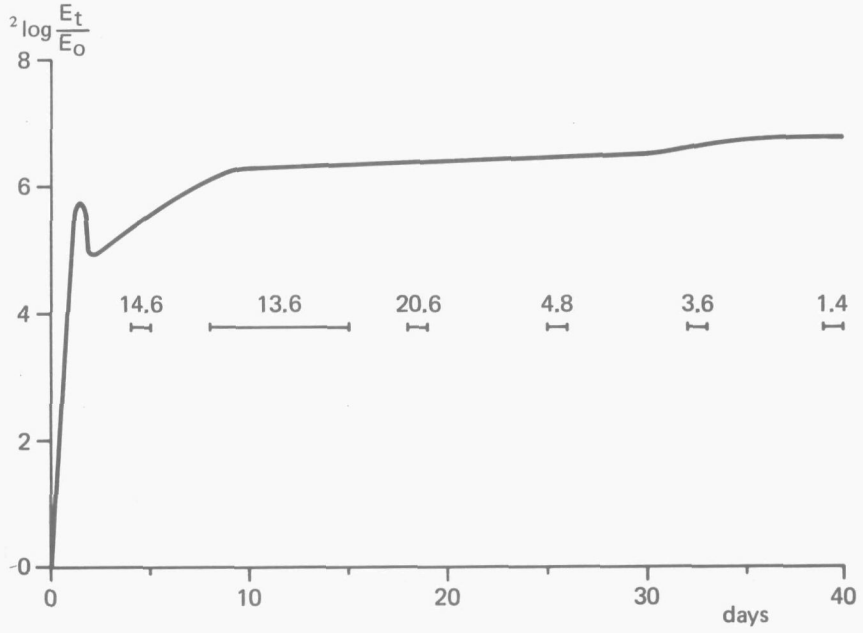


Fig. 4.12 Continuous culture of **B.pertussis,** strain 509; medium B-2; D =0.095 h^{-1}. The figures in the graph are mouse protective activities in IU per human dose of the effluent collected over the period indicated.

It is difficult to speculate about the nature of the observed phenomenon and about the frequency with which it is to be expected in further experiments. The fact that the results of potency testing become available only after four weeks, and the difficulty in avoiding the formation of dark aggregates led us to the decision to abandon the prospect of using continuous culture of **B.pertussis** for regular vaccine production.

In Table 4-12 an experiment is shown in which a semi-continuous procedure is performed. After outgrowth the culture is removed from the fermentor by air pressure, leaving about 25 ml as an inoculum for the next batch of medium introduced aseptically. No decrease in protective power is detectable, but cell yields increase. The fact that 22 transfers are made without the use of blood containing media (cf. *Rowatt,* 1957) or mouse passage (*Billaudelle* et *al.,* 1959) proves clearly that these two procedures are immaterial for obtaining good results.

SYNTHETIC MEDIA

A promising development has been made recently by *Stainer* (1970). His work at Connaught Laboratories is a continuation of the work of *Wilson*

Table 4-12 Semi-continuous culture of **B.pertussis.** Strain 509; Verwey medium; volume 8500 ml; ± 25 ml left as an inoculum. Mouse protective activity in IU per human dose; figures between brackets are independable.

Lot no.	Incub. time	Final pH	Cells x 10^9/ml	m.p.a.	Lot no.	Incub. time	Final pH	Cells x 10^9/ml	m.p.a.
1	53	8.00	35	13.8	12	32	7.75	48	-
2	42	7.95	31	11.6	13	40	8.35	56	3.6
3	48	8.10	29	(64)	14	48	8.40	56	-
4	48	8.00	31	4.0	15	48	8.02	53	2.6
5	48	8.00	29	3.4	16	32	8.12	56	-
6	48	8.00	31	(2.6)	17	40	8.40	59	4.0
7	48	8.00	34	4.0	18	48	7.80	59	3.0
8	48	7.95	31	5.4	19	32	7.85	59	5.6
9*)	48	7.75	28	-	20	40	8.40	78	3.6
10	48	8.05	31	-	21	48	8.40	59	-
11	48	8.00	39	8.4	22	48	8.25	59	4.4

*) Air-supply interrupted for some hours

Table 4-13 Composition of Stainer's basal medium ("GP") in mg/l.

Na-glutamate	10,700
l-proline	240
l-cystine	40
NaCl	2,500
KH_2PO_4	500
KCl	200
$MgCl_2.6H_2O$	100
$CaCl_2$	20
$FeSO_4.7H_2O$	10
Tris buffer	6,075
Glutathion	100
Ascorbic acid	20
Nicotinamide	4

pH: 7.6

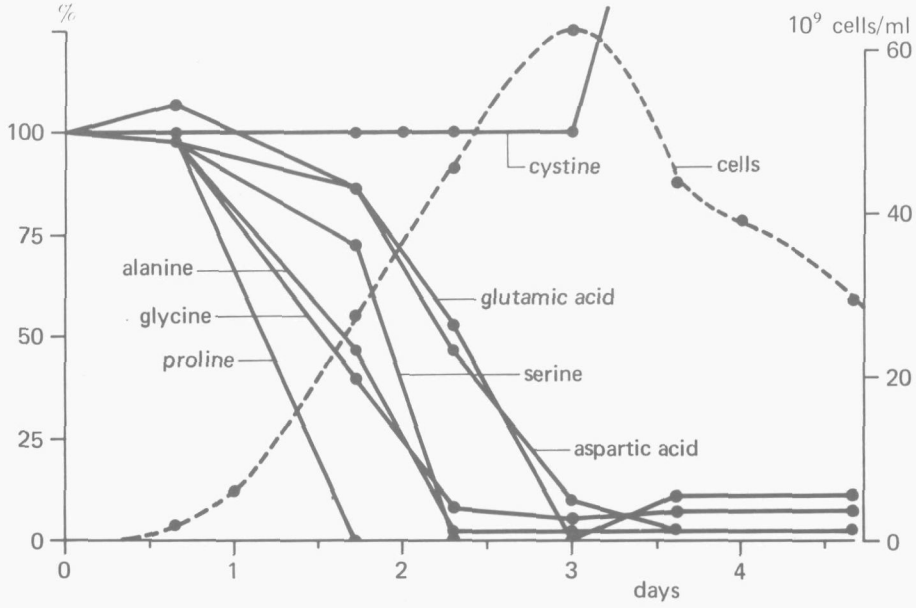


Fig. 4.13 The course of concentration of some amino-acids (day 0 is 100 %) in an oxygen controlled culture of **B.pertussis,** strain 509, pO_2 = 22 mm Hg.

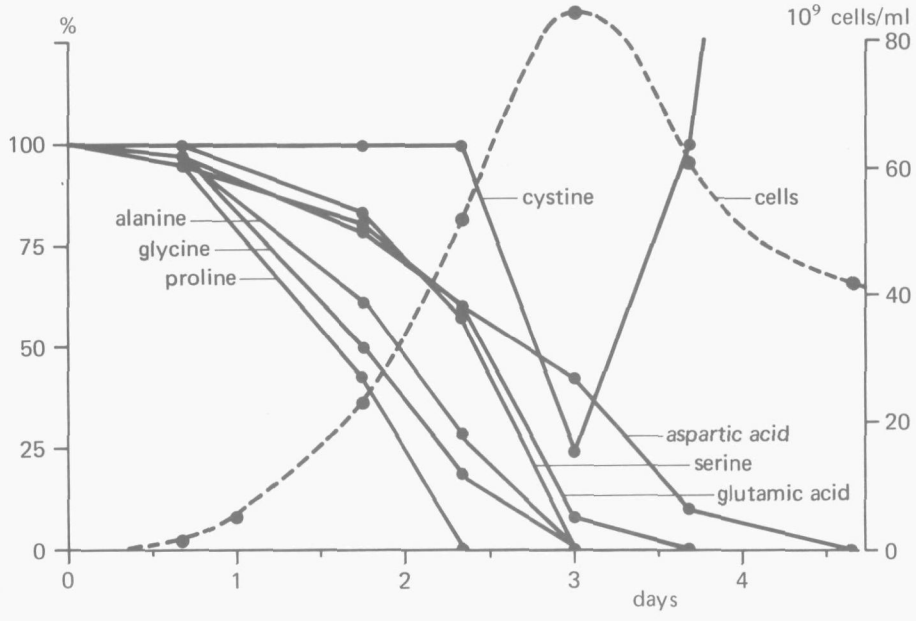


Fig. 4.14 The course of concentration of some amino-acids (day 0 is 100 %) in a culture of **B.pertussis,** strain 509, without pO_2-control.

(1963) and *Goldner* et *al.* (1966). The original defined medium of the latter authors contained 10 amino-acids, seven mineral salts and 14 "growth factors" such as liver coenzymes and nucleic acid derivates. The medium, although giving cells of satisfactory protective activity, was disregarded because of its low cell yield (5-10 x 10^9 cells/ml).

Stainer (loc.cit.) has simplified this medium, insofar as the basal formula (Table 4-13) contains only three amino-acids, six salts and three "growth factors". Increase of the concentrations of either glutamate or proline lead to cell yields of the same order (50-70 x 10^9 cells/ml) as with our B-2 medium. We have chosen the addition of glutamate rather than proline, because the former is cheaper (cf. formula 16 G.P.).

The importance of glutamic acid has been known for long. An impression of the importance of proline was already obtained by us through amino-acid analysis during growth; it showed (Fig. 4.13 and 4.14) that proline is the first amino-acid that is completely consumed.

A disadvantage already indicated by *Stainer* of the medium, is that a 5 % inoculum is needed for a good start, even when using precultures inoculated with cells from *Bordet-Gengou* medium (containing blood); this again shows that blood containing media are of no advantage. We confirmed that a minimal 5 % inoculum was required. This is an impractically large volume for seeding a 40 litre culture. Moreover, from our experience in almost all other cases, we expected difficulties with such an inoculum, being 5-10 times larger than usual (cf. p. 69).

Attempts to decrease inoculum size have been partly successful now. We found that a medium with 50 % of the "growth factors" was easier started. Finally it appeared that a medium with reduced oxygen tension (25 % of air saturation) also gave an easier start.

In Table 4-14 the results of a series of cultures in B-2 and *Stainer* medium are compared. Incorporation in a quadruple vaccine and extensive testing is

Table 4-14 Parallel cultures of **B.pertussis** in B-2 (B) or Stainer (S) medium (with starch); 40 litre cultures.

exp. no.	strain	medium	yield 10^9 cells/ml	m.p.a. IU per human d.
364	134	B	68	3.4
365	134	S	62	5.4
366	509	B	40	2.6
367	509	S	48	4.2

necessary to find out whether preparation of the pertussis vaccine on the latter medium, is justified.

ATTEMPTS TO PREPARE A FLUID VACCINE

In order to overcome undesired side reactions, ascribed to several cellular factors of **B.pertussis,** attempts have been made to solubilize the cells and to fractionate the solubilizate. Although initiated by us (*Van Hemert, Van Wezel* and *Cohen*, 1964) the work has been continued by *Nagel* (1970) and outlined in his thesis, to which one is referred. Experiments are in progress to combine two of the procedures proposed by *Nagel* (loc.cit.): treatment of the cell suspension with veronal buffer, followed by deoxycholate extraction. It is unknown whether this will lead to a product that is acceptable for human application.

DISCUSSION

It is not by chance that the cultivation of **B.pertussis** has been so extensively studied by us. Pertussis vaccine was the first one shown conclusively to be effective against the disease in man. Vaccination of infants is important because whooping cough is a serious threat to public health, and can only be prevented by vaccination. In the Netherlands the pertussis component is incorporated with polio vaccine, diphtheria and tetanus toxoids in a quadruple vaccine.

The pertussis vaccine was always produced by a relatively primitive cultivation method. The development work is also seriously handicapped by a time consuming and unreliable potency test. Furthermore, this is the vaccine to which most side-reactions caused by the quadruple vaccine are ascribed. This fact inspires, on the one hand, to look for changes that improve the vaccine, not only by the most natural although extremely difficult way of cell fractionation, but also by varying the cultivation method itself. On the other hand, there is always the risk that, if a modification has been chosen for better (or more efficient) production, in terms of cell or antigen yield, the final product will have changed to the worse as judged by side-reactions, stability or macroscopic appearance.

Although the B-2 medium has proven to give consistently good results, the *Stainer* medium, having a simpler composition, now looks promising. We have learned, however, to be cautious and are not yet able to advocate it unconditionally.

The dependence of antigen yield on the oxygen partial pressure in the culture during growth is a point that needs further investigation. It has been proven by following the concentration of several amino-acids during cultivation at different pO_2-levels, that a higher oxygen level leads to quicker

dissimilation of amino-acids. Moreover, it appeared that a single amino-acid, like glutamic acid, added in a later growth stage, was quickly dissimilated as long as the pO_2 was above zero. If some amino-acids are needed for the formation of the protective antigen, early exhaustion of these amino-acids would bring this formation to an early end.

The use of continuous culture provided a testcase for our apparatus, especially as the medium used is easily contaminated. The many experiments performed have helped us to develop the final form of many details of fermentor and auxiliary containers.

There was no evidence of unsuitability of continuous culture for pertussis vaccine production. The method might become attractive and widely applicable if a quick activity test (say 24-48 hours) were available, and a large quantity (say more than 10.000 litres per year) was required.

The acid precipitation method offered us the possibility, in the absence of a good aseptic continuous centrifuge, to introduce a more elegant method of further processing. Because several other laboratories had adapted the method, we did not reject it after the first negative findings. Further investigation showed that acid precipitation always led to an inferior vaccine, even though the vaccine passed the official tests. In a public health situation where every risk has to be avoided the final decision to abandon the method was clear.

Future work must include the development of a fractionated pertussis vaccine. A dependable consistent mass cultivation is then a prerequisite in view of the low over-all yields to be expected. A multiple of the present quantity could be produced in our plant, but this still leaves the further purification steps to be considered. If the veronal-treatment of *Nagel* were chosen as the first post-cultivation treatment, it could possibly be performed in the fermentor at the end of growth.

4.1.2 CHOLERA AND TYPHOID VACCINES

INTRODUCTION

There is a number of reasons to treat cholera, typhoid and paratyphoid vaccines in one section: they all have a comparable epidemiology, the infection being transferred from man to man through food and drinking water; they all cause serious illness of the gastro-intestinal tract. Furthermore, the causative organisms are all gram-negative bacteria, able to grow on relatively simple culture media; the methods of cultivation, and also of further processing are similar.

On some occasions the vaccines mentioned together with those from other gram-negative microorganisms such as **Shigella** and **Pseudomonas** are joined under the heading: "enterobacterial vaccines", as was the case at the International Symposium on Enterobacterial Vaccines, held in Berne in 1969 (cf. Symp.Ser.immunobiol.Standard., vol. **15** (1971)).

At present, there is serious doubt about the efficacy of immunization against infection by **S.paratyphi** A and B (cf. *Cvjetanović* and *Uemura,* 1965). Only recently *Hejfec* et *al.* (1968) claimed that a fivefold increase of the usual dose is effective in the case of paratyphoid B. For the time being, however, we have discontinued the application of both paratyphoid vaccines, as in the United States.

The causative organism of cholera was discovered by *R.Koch* (1886) and is now called **V.cholerae.** An endemic disease in India and Pakistan (Ganges delta) until 1817, it spread over the world and caused five pandemics in the 19th century. With improved sanitary arrangements, cholera is gradually restricted to its area.

Recently severe outbreaks, caused by **V.El Tor** have been reported; this organism is closely related to **V.cholerae,** giving the same symptoms and giving cross-immunity with the latter. The vibrios of both species multiply only in the intestine; the patients succumb often from dehydration through the intestines.

In 1892, *Haffkine,* a pupil of *Pasteur*'s prepared a vaccine, consisting of living cells, which was used in several parts of India. The conclusions of *Haffkine* regarding the effectivity of his vaccine were severly criticized. Later he recommended a vaccine, killed by heat at 50°C and preserved with 0.5 % phenol. *Kolle* was the first (1896) to introduce the killed vaccine and his name is still connected with the vaccine. Except for the method of cultivation, the present vaccine is still strikingly similar to *Kolle's.* For complete immunization two strains, Inaba and Ogawa, that are antigenically different, are necessary; the **V.El Tor** also exists in two antigenic types, similar to these Ogawa and Inaba types.

There are two new developments in vaccination against cholera; first there is the oral vaccine, consisting of large doses (up to 4×10^{10} germs) of live attenuated cells of **V.cholerae.** The initial tests on human volunteers by *Mukerjee* (1963) were promising.

Another approach resulted from the discovery of the cholera toxin, the so-called loop reactive substance. Although this substance which is rather thermolabile, has been detoxified by formalin, and used in small field trials in this form, no conclusions on the applicability for routine immunization can yet be drawn (cf. *Ungar* et *al.,* 1971). No research has yet been done in our Institute on either oral vaccine or cholera toxoid.

The typhoid bacillus was first described by *Eberth* in 1880, and isolated in 1884 by *Gaffky.* **S.typhi** is supposed to enter the body normally by the alimentary tract. Unlike **V.cholerae,** it is found in the blood during the first stage of the disease.

First vaccination against typhoid fever was done by *Wright* (*Wright* and *Semple,* 1897). The vaccine was prepared from ordinary strains. *Perry, Findlay* and *Bensted* (1933; 1934) concluded from experiments on mice that only fully virulent smooth strains could be relied upon to induce a satisfactory degree of immunity. As a result of the work of *Felix* and *Pitt* (1934) the vaccine (grown on agar and washed off with saline) was killed by heating for one hour at 53°C and preserved with 0.5 % phenol. Except for the cultivation method, the routine vaccine prepared in the R.I.V. is processed in the same way. Only recently has a vaccine, from acetone-dried cells which has given good results in extensive field trials, been advocated. It will be described in a separate section.

Activity testing of cholera and typhoid vaccine is difficult. Although mice are not normally infected by **S.typhi,** it is possible to challenge them after immunization by special methods, such as the addition of mucin. This method is used increasingly for the evaluation of typhoid vaccines; it should be kept in mind, however, that a direct correlation with efficacy has never been found in the field trials performed.

DEVELOPMENT OF CULTIVATION METHOD

There has been no difficulty in developing a medium, which permits good growth of **V.cholerae** as well as **S.typhi.** The composition is shown in Table 4-15.

Table 4-15 Basal medium for **V.cholerae** and **S.typhi** in g/l.

casamino acids (Difco Technical)	35
yeast extract (paste)	3
pH: 7.6	
glucose added during culture	

The basis for the cultivation method has come from the work of *Joó* et *al.* (1959; 1964) and from personal communications of *Geser* (Berne) and *Engelhardt* (Marburg). There is a tendency, especially with **V.cholerae,** to form a rough culture when all the glucose needed is added directly to the medium. Therefore the first experiments were directed towards establishing the appropriate rate of addition of glucose (as a 10 % w/v solution) to the culture.

V.cholerae as well as **S.typhi** form acid(s) from the glucose added. Therefore we first tried to couple the glucose addition to the pH-control. If, after addition of a little glucose to start the control, the pH was held at 7.6 with further automatic addition of glucose, growth rate and final cell yield were relatively low. It is known, with some bacteria, that the rate of continuous glucose addition can be reduced to the point at which almost no acid is formed (*Elsworth,* pers.comm.). This did not apply to **V.cholerae** and **S.typhi;** under such conditions they dit not show maximal growth.

Constant addition had the disadvantage that in the later stages of the culture, the demand for glucose was higher than the addition. The only satisfactory solution to the problem, although needing more frequent

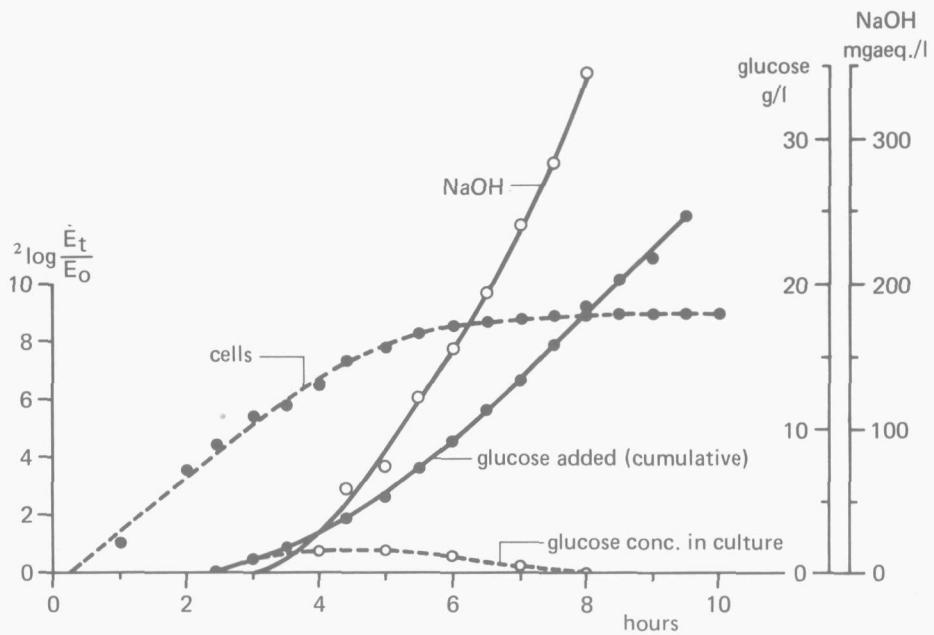


Fig. 4.15 Typical culture of **V.cholerae,** strain Inaba.
7 litre culture; air flow rate 3 l/min; pH-control at 7.6.

supervision, was by increasing the rate of glucose addition stepwise during cultivation.

A typical culture of **V.cholerae**, in which the glucose addition was increased from 1 to 4 g/lh, is shown in Fig. 4.15. A little excess of glucose is found in the culture until the end of growth, proving that the addition rate is suitable to keep the concentration in the culture low. The doubling time in the logarithmic phase is about 40 minutes and fairly constant from one culture to an other. Cell yields are around 30×10^9 germs/ml (corresponding to 1.5 g dry weight per litre).

Occasionally there was spontaneous precipitation in samples taken during cultivation from batches of **V.cholerae** prepared along these lines, especially with the Inaba strain. In most cases the phenomenon was transient and at the end of growth a normal smooth culture was present. In addition, plating out of such a sample gave rise to characteristic smooth colonies. It was postulated that partial oxidation of glucose could give rise to accumulation of organic acids causing the rough-like behaviour of the cells. Therefore the problem was assessed from two sides: decrease of glucose addition rate and establishment of oxygen control.

Table 4-16 shows experiments with oxygen control at 10 % air saturation, and also without oxygen control. Fig. 4.16 shows a culture with oxygen control and a glucose addition increasing during cultivation from 0.6 to 2.0 g/lh. The increase in final cell yield caused by the oxygen control is great: about a factor 3. Moreover, no rough behaviour has occurred subsequently.

Table 4-16 **V.cholerae**, strain Inaba, cultivation with and without O_2-control at 16 mm Hg; 7 litre culture; pH-control at 7.6.

Exp. no.	O_2-control	glucose g/lh		yield 10^9 cells/ml
		from	to	
39	without	1.4**	3.5	37
40	with	0.7	2.2	109
41	without	0.7*	3.3	26
42	with	0.3*	1.1	81
43	without	1.5	3.6	27
44	with	0.6	2.0	88

* increase right after start because of decrease in growth rate

** transient rough-formation

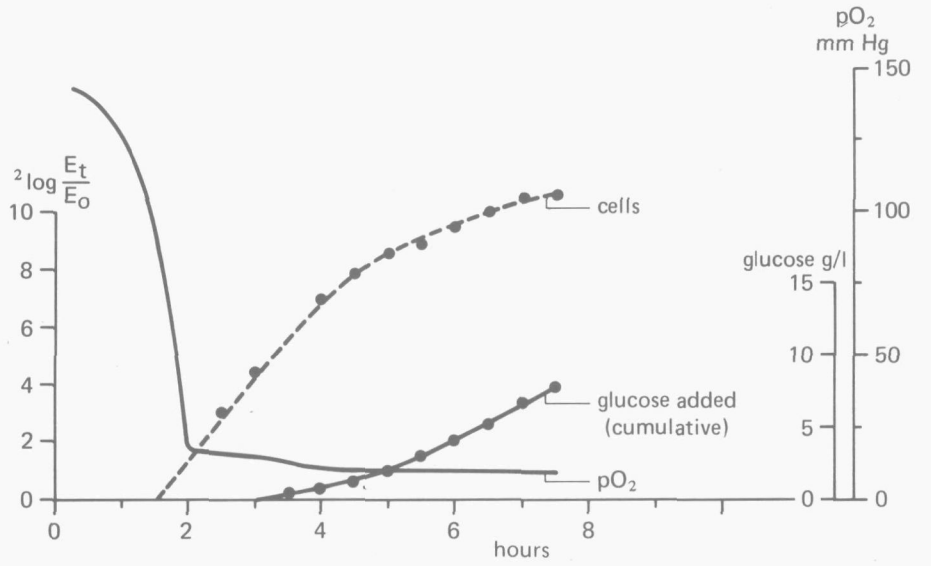


Fig. 4.16 Culture of **V.cholerae** with pO_2-control at 15 mm Hg, and pH-control at 7.3.

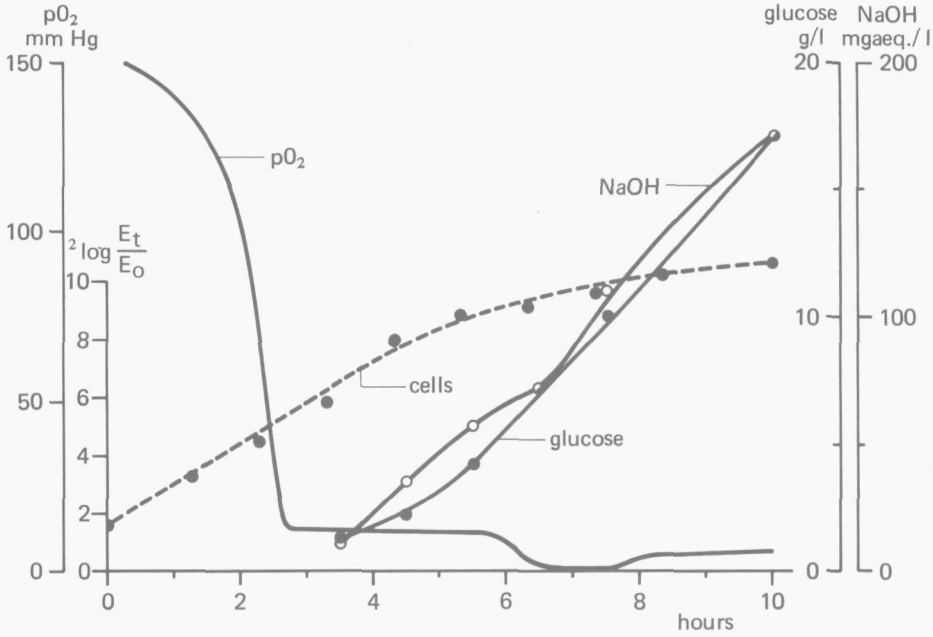


Fig. 4.17 *Culture of* **S.typhi,** strain Ty-2 with pO_2-control at 15 mm Hg. 7 litre culture; gas flow rate 3 l/min.; stirrer speed 450 r.p.m. Glucose concentration in graph is the cumulative value of glucose added.

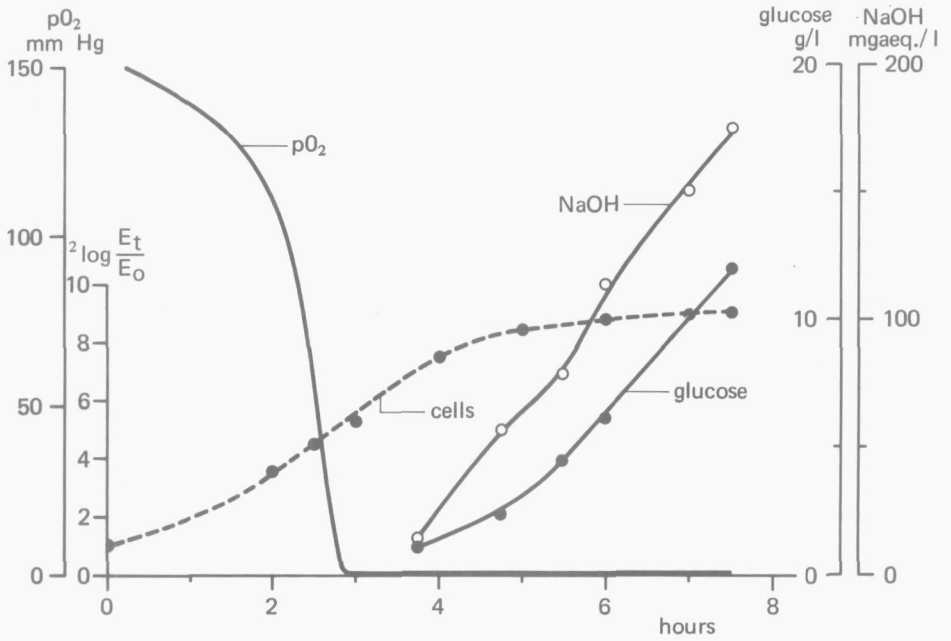


Fig. 4.18 Culture of **S.typhi,** strain Ty-2, without pO_2-control. 7 litre culture; air flow rate 3 l/min.; stirrer speed 450 r.p.m. Glucose concentration in graph is the cumulative value of glucose added.

The cultivation of **S.typhi** has been similarly developed. The strain used is Ty-2, (*Felix,* 1941), accepted internationally following the field trials described by the *Yugoslav Typhoid Commission* (1964) and the *Typhoid Panel of the U.K. Department of Technical Cooperation* (1964) in British Guiana. An addition of glucose increasing from 1.5 to 4.3 g/lh and a pH-control at 7.6 gives cell yields of about 40×10^9 germs/ml, corresponding to about 1.5 g dry weight/l (see Fig. 4.18). An initial experiment with pO_2-control at 15 mm Hg together with pH-control at pH 7.6 led to a more than threefold increase in cell yield (4.8 g/l) (see Fig. 4.17).

It is notable but unexplained, why *Benenson* et *al.* (1964) in their preparation of the acetone-dried vaccine, still make use of an agar surface culture in Kolle flasks.

The data obtained from regular cultures of **V.cholerae** and **S.typhi** were used to make a rough calculation on the dissimilation of the glucose. For **V.cholerae** the NaOH addition to maintain the culture at pH 7.6 is 20-25 mg equivalents per gram glucose added. For **S.typhi** this figure is about 15.

However, **V.cholerae,** unlike **S.typhi,** gives off carbon dioxide: the amount, estimated from gas analysis in oxygen controlled cultures is 5-10 mmol per g glucose. Although these figures give no accurate basis for calculations, it can be concluded that, in **V.cholerae,** glucose is broken down to carbon dioxide and acids, with an average of 1 C atom per acid group. In **S.typhi** no gas is produced, but only acids, with an average of 2 C atoms per acid group.

ACETONE-DRIED TYPHOID VACCINE

Benenson et *al.* (loc.cit.) prepared typhoid vaccine in two ways: inactivation by heat in the presence of 0.5 % phenol, and drying in an acetone suspension. Field trials in Yugo-Slavia and British Guiana have proven that the so-called acetone-dried vaccine was the most efficacous one.

The method consists of the addition of an excess acetone to a suspension of **S.typhi** in saline. After decanting, an other volume of acetone is added; the procedure is repeated several times. The final suspension in acetone is inactivated by heating at 37°C for 24 hours. In the original procedure the suspension was diluted with a mixture of acetone and carbon tetrachloride to a concentration of 25×10^9 cells per ml and dispensed in vials for lyophilization.

The preparation of the acetone suspension gave no problems. The main difficulties have been encountered in the preparation of the final suspension for lyophilization. Here it is very important that a finely dispersed stable suspension is achieved. Otherwise reproducible dispensing of quantities of 0.4-1.0 ml in vials is not feasible.

The original procedure with the acetone-tetra mixture gave a coarse suspension, and the method was discarded.

Three alternatives were tested:

a. suspension in acetone,
b. suspension in equal volumes of acetone and saline,
c. suspension in saline.

The results are shown in Table 4-17. They indicate that only alternatives b and c are feasible; b is preferred as being closer to the original method and because the solvents will evaporate quicker than in the case of c during lyophilization.

DISCUSSION

The lack of reliable potency tests meant that developments could only be directed towards the improvement of the cultivation method, but without diverging greatly from techniques employed elsewhere for vaccines, showing good protection in field trials.

Table 4-17 Preparation of acetone-dried typhoid vaccine. Influence of resuspension fluid on distribution in ampoules. Dispensed volume determined by weight. 90 % interval indicates the limits between which 90 % of the volumes lie. Cell concentration determined by opacimetry; in the dried samples the mean dispensed volume (in the same column) is used for calculation.

combination		a	b	c
suspension fluid	% acetone % saline	100 0	50 50	0 100
density		0.79	0.95	1.00
mean of dispensed volume		0.554	0.568	0.568
90 % interval		0.53-0.57	0.54-061	0.53-0.62
before drying				
cell conc. 10^9/ml		13.6	10.4	7.8
90 % interval		10.1-17.7	9.6-11.8	7.4-8.3
after drying in vacuo				
cell conc. 10^9/ml		8.8	8.5	8.2
90 % interval		3.9-18.0	7.1-9.7	6.6-9.3
after drying in N_2				
cell conc. 10^9/ml		9.1	8.0	8.4
90 % interval		6.3-11.1	6.6-10.3	6.6-10.3

Control of pH as well as pO_2 gives rapid growth and high cell yield in **V.cholerae** as well as **S.typhi.** In typhoid vaccine production, a development, introduced in the U.S.A., leading to a dried product with expected high stability, has been followed. It now looks promising.

Continuous culture is not necessary for the preparation of these vaccines, because a few batches make a years' requirement for the Netherlands. To our idea, there is even no obvious necessity to use continuous cultivation for countries in which, through a high population, together with unsatisfactory sanitation standards, great quantities of vaccine are needed. It cannot be foreseen which quantities will be needed if oral vaccination against cholera and typhus needing 10-100 times more material would come into use. Continuous cultivation will perhaps then prove advantagious.

4.1.3 B.C.G. VACCINE

INTRODUCTION

For immunization against infection with **Mycobacterium tuberculosis** a preparation called **B.C.G.** vaccine is used. That the tubercle bacillus is the one essential cause of tuberculosis has been shown conclusively by *Koch* in 1882 in his classical study. The organism had escaped attention for a long time due to its difficulty to stain, and its very slow growth, making detection on agar plates difficult.

The infection by **M.tuberculosis** that takes place mainly through the different mucous membranes of the body, not only has its foothold in the lungs; also other parts of the body, such as the bones and joints, and the genito-urinary tract may be affected. Whereas the pulmonary infection in man is almost exclusively caused by the human type, part of the non-pulmonary lesions are caused by the bovine type.

As early as 1897 *Koch* tried to develop a vaccine by killing a suspension of tubercle bacilli by several agents such as heat, acids, alkalis, formalin etc. Neither he nor later workers have been able to obtain a promising vaccine this way.

On the assumption that acquired immunity depends upon the persistence of living bacilli in the body, *Calmette* introduced a live vaccine of a bovine strain that had become avirulent as the result of several years subculturing on a glycerol-bile-potato medium. This strain was called (1921) the **Bacille Calmette-Guérin.** Its immunogenicity has been confirmed in animals, and in some field trials with humans.

An extract of tubercle bacilli, prepared by heating, concentrating and then filtering the grown culture was prepared by *Koch* and called tuberculin. It is used as a diagnostic agent, either in its original form (old tuberculin) or after ultrafiltration and trichloroacetic acid precipitation (P.P.D. = Purified Protein Derivative, *Seibert* et *al.,* 1934). It gives, when injected intradermally in a very dilute form, a measurable skin reaction in men and animals that have been infected with the bacillus.

The efficacy of the **B.C.G.** vaccination has been proved. In the Netherlands, the number of infections with tubercle bacilli is low. Vaccination is, therefore, only recommended for children in tuberculous families and for specially exposed groups such as nurses and medical students.

M.tuberculosis grows like most mycobacteria normally as a coherent pellicle, also on the surface of fluid media. The method of cultivation that has maintained itself up to the present is in glass flasks on a shallow layer of Sauton medium, containing inorganic salts, citric acid, asparagin and glycerol.

Dubos and *Middlebrook* (1947), in their attempts to constitute a medium for disperse growth of **M.tuberculosis,** added Tween-80 (polyoxyethylene derivative of sorbitan mono-oleate) as a dispersing agent. It contains free oleic acid, which is toxic for growth, and therefore subsequently they also added bovine albumin, to neutralize this effect. *Dubos* and *Fenner* (1950) ommitted glycerol and glucose from the medium because these components, although giving higher cell counts, caused rapid loss of viability during prolonged cultivation.

Ungar, Muggleton and *Dudley* (1962) applied this finding of *Dubos* and *Fenner* to their medium. They claim that the omission of glycerol enables the **B.C.G.** cells to survive the freeze-drying process to a much greater extent. Instead of Tween-80, and its neutralizing agent bovine albumin, they incorporated Triton WR 1339 in the medium. It will be shown in the next section that through replacing Triton by Tween-80 in this medium neither growth nor viability were impaired, but the average size of the cell clumps diminished considerably. For composition of media, see Table 4-18.

The classical method of preparation of vaccine from a culture grown on Sauton medium consisted of pressing the bacterial pellicle in a *Birkhaug* apparatus to a humid cake (containing approximately 20 % dry weight of cells) and preparing a suspension by shaking in the presence of steel balls.

Table 4-18 Composition of the fluid **B.C.G.** media used in g/l (unless otherwise stated).

	Dubos		Ungar	
KH_2PO_4	0.95		1.0	
Na_2HPO_4. 12 aq.	5.97		5.0	
l-Asparagine	1.90		4.0	
Casitone, Difco	1.90		1.0	
Ferriammonium citrate	0.004 75		0.1	
$MgSO_4$. $7H_2O$	0.009 5		0.125	
$CaCl_2$. $2H_2O$	0.000 475		0.001 32	
$ZnSO_4$. $7H_2O$	0.000 095		0.000 89	
$CuSO_4$. $5H_2O$	0.000 095		0.000 78	
Albumin, bovine	20		-	
Glucose	5		-	
Monosodium glutamate	-		4.0	
l-glutamine	-		4.0	
NaCl	0.765		-	
Tween-80 (Atlas)	0.5	ml	0.5	ml
or Triton WR 1339 (Winthrop)	0.25	g	0.25	g

The cell material from a deep culture can be directly resuspended after centrifugation. The deep culture method, in the original non-stirred version, has become the method of choice to prepare starting material for lyophilization. Through this procedure, the half life of this – living – vaccine is increased from one week at 20°C to about one month at 37°C.

DEVELOPMENT OF CULTIVATION METHOD

No data from literature were available on the possibility of cultivating the **B.C.G.** organism in homogeneous culture. There was no reason, however, to doubt whether this could be performed with the medium of *Ungar* et *al.*, mentioned above.

The strain used was received in 1962 from the "Institut Pasteur" in Paris as 1173-PZ. The basal medium (without detergent) was spray-dried and provided a constant starting material for the experiments.

During cultivation it was seen that too vigorous stirring was detrimental, a stirring rate of 400 r.p.m. in a seven litre culture causing damage. However, a rate of 200 r.p.m. provided good mixing and, as pO_2-measurement showed,

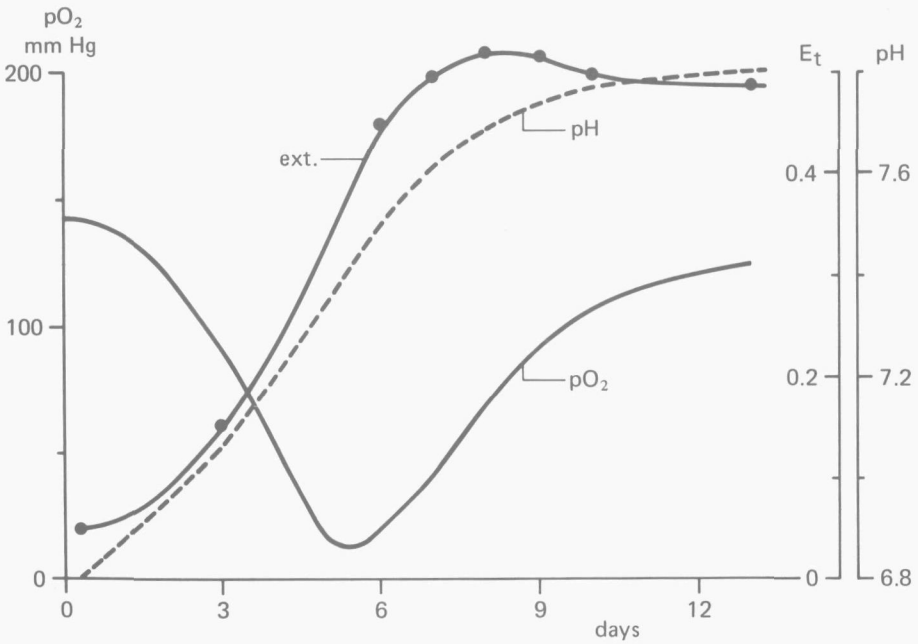


Fig. 4.19 The course of extinction, pH and pO_2 (dissolved) in a typical homogeneous culture of **B.C.G.** 7 litre culture in Ungar medium with Tween-80 instead of Triton. Air flow rate 3 l/min.

never brought the culture to oxygen starvation. This is mainly due to the low growth rate: normal doubling times are in the order of 24-48 hours.

The original method of preparing the seed culture consisted of three subsequent subcultures in *Löwenstein* medium followed by three in *Dubos* medium and one in *Ungar* medium. This number of steps may be reduced considerably and experiments to achieve this are under way.

Fig. 4.19 shows the course of growth and pO_2 during a typical cultivation in the medium with Triton. pO_2 shows a minimum, at about the end of logarithmic growth. Harvesting shortly after this point gives good results, as confirmed in the results of the *Warburg* tests (v.i.). The harvest of two of these cultures, and the harvest of static cultures in the same medium, were processed in the routine way for vaccine production. This involves centrifugation and resuspension in a medium containing, besides glucose and Tween-80, Haemaccel (a modified gelatin, intended for intravenous use, produced by Behringwerke). After the required dilution the menstruum is lyophilized in small portions.

Comparison of preparations from static and from stirred cultures, on the basis of the determination of "viable units" (by colony counting on *Löwenstein* medium) appeared to be impossible, because suspensions of **B.C.G.** always consist of clumps, with the clump size varying in an undefined and uncontrollable way.

In Table 4-19 the ratio of $\frac{G}{E}$, i.e. the viable units/ml divided by the extinction values, is given for homogeneous cultures in medium with Triton c.q. Tween. Although E is only 20 % higher, the viable count is 10 times higher in the latter suspension agent. After experience had been gained with microscopic estimation of the number of cells per clump (*P.Smid*) it appeared that the clumps in the Tween medium were smaller by roughly a factor of 10.

Extinction values were, in all cases, directly proportional to dry weight. This is shown in Table 4-20 for a number of consecutive batches; although

Table 4-19 Ratio of G/E and clump size in cultures of **B.C.G.** in Ungar medium with Triton or Tween. G = viable count on Löwenstein medium in 10^6 per ml. E = extinction at 650 nm at the end of growth. Figures given are the mean of three experiments; the clump size is a microscopical estimation.

medium with	E	G/E	clump size
Triton	0.400	20-40	100
Tween	0.500	160-350	10

Table 4-20 Relation of extinction, dry weight and viable count, measured at the end of growth in a series of **B.C.G.** homogeneous cultures. Ungar medium with Triton.
E = extinction at 650 nm.
W = cell dry weight in g/l.
G = viable count on Löwenstein medium in 10^6 per ml.

Exp.no.	extinction E	dry weight W	W/E	germcount G	G/E
47	0.450	0.277	0.62	18.5	41.1
50	0.485	0.276	0.57	14	28.9
51	0.505	0.356	0.70	23/41.5	45.5/82
52	0.465	0.298	0.64	19.2	41.3
53	0.490	0.331	0.68	14	28.6
54	0.430	0.308	0.72	19.2/32	44.7/74

the ratio $\frac{G}{E}$ is varying by a factor of 3, the ratio $\frac{W}{E}$ only shows differences of 25 %, well within the limits of accuracy. So further development, that is now in progress, will be guided no longer by viable count, but by extinction values. The somewhat paradoxical use of a method, determining live as well as dead cells in standardizing a live vaccine is justified as follows:

1. Standardization of the process guarantees a minimal variation in the relative amount of living cells.
2. Use of the "viable count" as a qualitative check on viability of a suspension diluted according to extinction c.q. dry weight.
3. Introduction of the *Warburg* method as a measure of viability.

The *Warburg* method has proven in preliminary experiments to give dependable results.

Table 4-21 lists values of oxygen consumption in a *Warburg* apparatus of samples from a regular homogeneous culture. In order to minimize the sample volumes, a micromethod has been chosen. With this method 1 ml is used. A sample of 20 ml is taken from the culture every 12 hours. After centrifugation the sediment is taken up in 4 ml Sauton medium; this enables triplicate determinations. KOH is used in the centre well and the determination lasts from 30 to 60 minutes dependent upon the oxygen consumed. Results are expressed in μl O_2 per 5 ml of original culture. The values were seen to increase with time. There is a maximum, about 12 hours before the maximum of cell density is reached. The oxygen consumption value, expressed per cell not per culture volume, drops sharply after this

Table 4-21 The course of pO_2 (dissolved), and the oxygen uptake in samples of a culture of **B.C.G.** 7 litre culture in Ungar medium with Tween-80; air flow rate 3 l/min; stirrer speed 200 r.p.m. Oxygen uptake measured as described in the text, and expressed as μl O_2 per 5 ml of original culture per hour. An approximation of the oxygen uptake per cell (quotient of column 4 and 2) is given in the last column.

Cultiv. time, h	Extinction E_t	pO_2 diss. mm Hg	O_2-uptake μl	O_2-uptake "per cell"
0	0.013	143	-	-
36	0.070	136	-	-
46	0.090	96	6.7	75
57	0.134	71	9.3	69
70	0.187	32	12.4	67
81	0.250	(11)	14.7	59
94	0.310	(41)	16.0	52
105	0.350	26	21.0	60
118	0.415	23	26.3	63
129	0.450	33	21.0	47
142	0.450	47	22.0	49
152	0.435	68	18.2	42

time. The figure also shows that the minimum of dissolved pO_2 (in practice 12-24 hours later) gives a cell suspension with a high vitality. The small decrease of the oxygen consumption value per cell in the growth phase is perhaps due to a gradual increase of dead cells, which are counted with the living cells in the opacimetric determination.

DISCUSSION

B.C.G. is the slowest growing of the bacteria studied and is the only one that gives serious trouble in homogenization. This is the more frustrating because W.H.O. requirements still recommend a viable count, and count values depend upon the clump size, which appears to vary in an uncontrollable way.

In respect to human application **B.C.G.** vaccine stands apart from the other vaccines studied. The efficacy depends upon a certain amount of multiplication in the human body. This means that special care should be taken to harvest the culture on the moment of maximal viability, and that processing and storage is directed towards maintaining a high viability.

Homogeneous cultivation in the presence of detergents has proven

possible. Of these Tween-80 gave the smallest clumps; the simultaneous addition of bovine albumin (to counteract free oleic acid from the Tween), suggested by *Dubos* has not been found necessary. We have substantiated the view of *Dubos,* and also of *Ungar* in respect to the addition of carbon sources such as glucose and glycerol: besides giving a high cell yield they cause an increase in death rate. In addition the **B.C.G.** cells grown in a medium without glucose or glycerol survive lyophilization better.

The relation between clumping and viable count must be considered. Clumping size can vary in an uncontrollable way, up to a factor of 10; viable counting is thus almost useless for standardization. Measurement of the extinction value however gives a good correlation with bacterial mass, irrespective of clump size. If, as achieved by harvesting at a point related to the minimal pO_2, the process is reproduced as accurately as possible, the percentage of living cells is supposed to have but small variation, and at any rate less than the factor 10 with which the clumping size is known to vary. This means that omission of the viable count would give a sounder basis to the consistency of the vaccine.

Introduction of the *Warburg* technique for measurement of oxygen consumption by the cell suspension is now becoming widely used to test viability. We have shown that, during cultivation, this value is correlated with the dissolved oxygen tension in the culture, and reaches a maximum shortly after the latter reaches its minimum.

Field trials have been performed in Spain with batches of lyophilized **B.C.G.** obtained from static and from homogeneous cultures (*Sybesma* and *Bleiker,* 1971). The latter were in no respect inferior: tuberculin conversions were of the same order and side-reactions not higher than of the non-homogeneous vaccine. So the introduction of the homogeneous cultivation for **B.C.G.** vaccine production, as being simpler and more reproducible, is strongly recommended.

4.2 Toxoids

The vaccines to be described under this heading are toxins which are excreted by the bacterial cell and have undergone a treatment to destroy their toxic properties, leaving intact the immunogenic activity. This treatment, called detoxification, is commonly effected by addition of formaldehyde to modify the toxin molecule into a compound (or compounds) called toxoid. The reaction of toxin (or toxoid) with the antitoxic serum which contains antibodies elicited by the corresponding toxin, offers a quick and easy assay method (*Ramon* flocculation test) to follow the course of toxin formation during the cultivation. The result is expressed in flocculation units per ml (Lf/ml). The rapidity of this test (1-2 hours), compared with the biological tests of the particulate vaccines (weeks), is one of the reasons why the production of toxins is discussed separately.

All the toxins considered are exotoxins. These exotoxins have a characteristic pharmacological effect on the susceptible host and are usually fatal in a very small dose. They give rise to specific antibodies and are rather thermolabile; chemically they are proteins. The toxicity of endotoxins, compared with exotoxins, is of a lower order of magnitude. Endotoxins are, moreover, more thermostable, and are generally considered not to form specific antibodies; chemically they mostly contain, besides protein, lipo--polysaccharide moieties.

Although, as the name implies, exotoxins generally are more easily excreted from the cell than endotoxins, they differ among themselves in their excretion pattern. *Raynaud* et *al.* (1954; 1955) distinguish three groups of exotoxins (called "toxines holoprotéiques" or "toxines protéiques" by them):

1. Toxins, bound firmly to the cell, only being freed from the cell by means of special procedures. Examples are the neurotoxin of **Shigella shigae** and the toxin of **Pasteurella pestis.**
2. Toxins, diffusing freely into the culture fluid during the growth phase, intra- and extracellular concentrations being always of the same order of magnitude. Diphtheria toxin and the α-toxin of **Staphylococcus aureus** belong to this group.
3. Toxins that, although diffusing into the culture fluid, build up a relatively high concentration in the cells during growth. It is only in the later stages, or after cessation of growth that intra- and extracellular

toxin concentrations become equal. Many clostridial toxins, including tetanus toxin, belong to this group.

The production of toxins may be seen as a bacterial culture excreting a cell substance into the culture fluid. The following examples show that they are easier accessible from a kinetic point of view than those of the foregoing group of particulate vaccines. They are also more accessible for purification procedures. A widely used method for purification, performed either before of after detoxification, is by ultrafiltration to increase concentration followed by fractionated ammonium sulphate precipitation (see *Van Ramshorst,* 1951; 1963). Detailed treatment of these procedures will not be delt with here.

4.2.1 DIPHTHERIA TOXOID

INTRODUCTION

Until recently diphtheria has been a contagious disease of great epidemiological importance and was considered to be one of the major threats of the health of children. The bacterial cause of diphtheria was shown by *Löffler* in 1884 to be a microorganism, now called **Corynebacterium diphtheriae.** Inoculation of a pure culture of this organism, isolated from the throat of a person suffering from clinical diphtheria, caused death after a few days in several species of experimental animals. Recovery of the organism, however, was only possible from the site of infection and not from lesions in the internal organs. Later on (*Roux* and *Yersin,* 1888) it was found that sterile culture filtrates caused the same symptoms in animals. It was concluded that an extracellular toxin was responsible for the clinical sequelae of infection, and for death, by the direct action of the toxin on the heart muscle.

Von Behring and *Wernicke* (1892) showed that active immunization of susceptible animals could be effected by inoculating them with increasing doses of living culture after a protective dose of antitoxin serum. *von Behring* (1913) was the first to vaccinate children against diphtheria, using a toxin-antitoxin mixture. Such a mixture of a toxic culture filtrate with a slight underdose of antitoxic serum has in some instances, however, caused toxic reactions and death (cf. *Brandwijk,* 1926). An important step was made by *Glenny* et *al.* (1921; 1923) and *Ramon* (1923; 1928) who found that the diphtheria toxin could be altered by formalin in such way that it is deprived of its toxicity without destroying its antigenic power. The product obtained by the formalin treatment is called toxoid, or anatoxin in French speaking countries. Complete toxoiding, resulting in disappearance of toxicity, is checked by inoculation in guinea-pigs or rabbits.

In the early years of diphtheria toxin production (1928–1946) the yield was too low (3-10 Lf/ml) to permit purification methods to be carried out. *Holt* (1947) was the first to purify the toxoid; he adsorbed the purified product onto aluminium phosphate. In our Institute the purification is still performed according to *Van Ramshorst* (loc.cit.), by ultrafiltration and fractionated ammonium sulphate precipitation.

Normally the diphtheria toxoid is administered as a triple vaccine, combined with pertussis vaccine and tetanus vaccine, or as a quadruple vaccine containing polio vaccine in addition.

In the thirties, 1000-2000 cases of diphtheria occurred per year in our country. In the second world war about 250,000 cases, with about 30,000 deaths were recorded. Due to the introduction of mass government vaccination (1953) this has been reduced to almost zero, cf. the survey by *Tasman* (1964).

DEVELOPMENT OF CULTIVATION METHOD

At the onset of our experiments the cultivation of **C.diphtheriae** was the most advanced one in respect to homogeneous culture. *Linggood* and *Fenton* (1947) developed a fluid medium (Table 4-22) containing, among others, a papaic digest of horse meat and maltose. In the aluminium fermentors (design of Wellcome Research Laboratories) regular toxin yields of 200 Lf/ml had been obtained. Aluminium tanks were chosen, because excess iron is detrimental to toxin production (v.i.). Transition, however, to the new glass fermentors with stainless steel lid, shaft and tubing did not impair toxin yield. Evidently the critical Fe-concentration (v.i.) is not reached this way.

The influence of papaic digestion of meat on the toxin production was our first study. Variations in toxin yield could be attributed to differences in quality of the papain batches used. It was supposed that oxidation of the enzyme during storage accounted for the deterioration. Addition of 0.002 M cysteine to the digestion fluid, containing 1.5 g papain per litre medium, gave good results with all papain batches used, cf. Tables 4-23 and 4-24. The

Table 4-22 Composition of basal culture medium for **C.diphtheriae** in g/l.

Beef meat*, approximately	155 g
Yeast extract (Gistex-paste, Delft)	0.15 g
Sodium lactate 60 % w/v	1.5 ml
Maltose 50 % w/v**	50 ml
$MgSO_4.7H_2O$	0.62 g
β-alanine	1.75 mg
nicotinic acid	1.75 mg
pimelic acid	0.11 mg
$CuSO_4.5H_2O$	0.75 mg
$ZnSO_4.7H_2O$	0.60 mg
$MnCl_2.4H_2O$	0.22 mg

pH : 7.8

* Digestion of meat involves the approximate amounts of the following compounds:

Papain	1.5 g
NaOH 50 % w/v	8 ml
HCl 35 %	2.5 ml
acetic acid, glacial	6.0 ml
cysteine hydrochloride	0.25 g

** In some experiments replaced by glucose.

Table 4-23 Influence of addition of cystein during papaic digestion on yield of diphtheria toxin.
L - 10: medium in which, during preparation, papain batch no. 3 is used without cystein.
L - 13: the same, with 0.002 M cystein added in the digestion fluid. In both cases 154 g papain per 100 l medium was used.
Toxin titres in Lf/ml reached after ± 50 h cultivation (mean of two batches).

medium	toxin yield
L - 10	90
L - 13	270

Table 4-24 Correlation of enzyme-activity of papain (without cysteine and EDTA) used for medium preparation, with yield of diphtheria toxin in Lf/ml.
The toxin-figures given are averages of at least two batches. Enzyme-activity measured with papain and EDTA is given as a comparison.

Papain batch	Anson-units per mg papain		Toxin yield
	with	without	
1	4.1	0.7	250
2	4.2	0.6	180
3	4.6	0.4	100

determination of enzyme activity of papain was performed on a substrate of denaturated hemoglobin. The peptides formed are dissolved in trichloro--acetic acid and measured at 280 mμ in a spectrophotometer, with tyrosine as a standard. In the original prescription, (kindly made available by the Biochemical Laboratory of Organon, Oss) 0.03 M cysteine and 0.006 M EDTA were added. This appeared to eliminate the differences in the papain batches. Omission of both reagents gave enzyme activities correlating with the toxin yields of the media prepared with the use of these batches.

In experiments in which air flow rate and stirrer speed were varied, it was found that toxin yields were, within wide limits, independent of aeration conditions (*Van Hemert* and *Van Wezel,* 1966; *Van Wezel* and *Van Hemert,* 1967a). There were indications that a minimum of aeration is required for good toxin production. In experiments with very low level of aeration, toxin

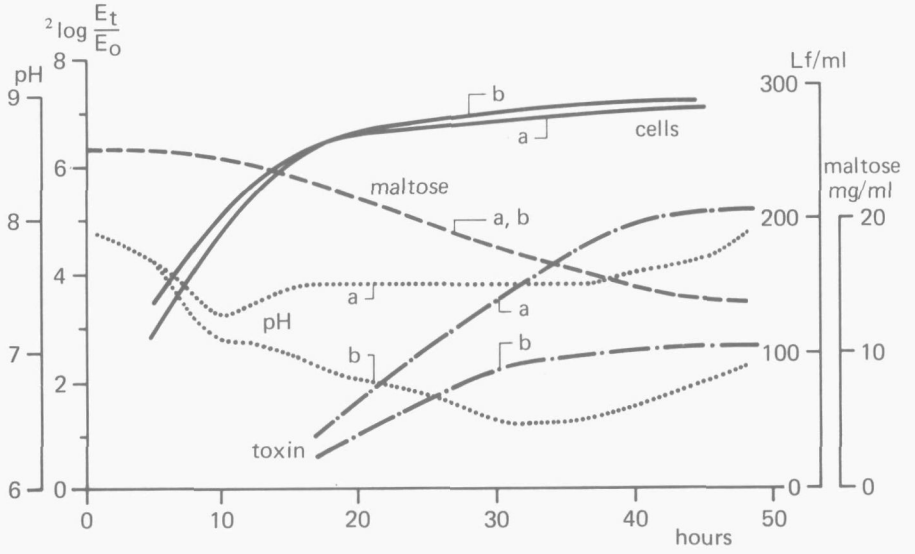


Fig. 4.20 Influence of air flow rate on toxin production and pH by **C.diphtheriae.** 6.5 litre cultures in modified Linggood-medium; stirrer speed 650 r.p.m. Air flow rates: a: 1 l/min; b: 0.25 l/min.

formation is impaired, although growth itself may be only slightly influenced. In Fig. 4.20 two cultures are compared, differing only in the amount of air supplied. The growth curve is similar in both cases, but toxin production is low with the lower aeration rate. This confirms the findings of *Raynaud, Alouf* and *Mangalo* (1959) and also of *Edwards* (1960) that no simple correlation exists between the amount of growth and the amount of toxin produced. *Pappenheimer, Miller* and *Yoneda* (1962) found that toxin is produced during the terminal stages of growth, whereas *Raynaud* et *al.* (loc.cit.) had already established the fact that there was no detectable concentration of toxin in the cells throughout the growth cycle.

However, there is another phenomenon which seems to affect the quality of the culture in respect to toxin production. As can be seen in Fig. 4.20, the course of pH in the culture with low production differs distinctly from the pH curve in the culture with normal production. This phenomenon has been widely found. Whereas with normal production the pH levels off between 7 and 8, in low production the pH falls to values between 6.0 and 6.5.

In the initial experiments there seemed to be a correlation between toxin formation and maltose consumption, but later comparative batches did not confirm this finding; this is in accordance with *Edwards* (loc.cit.) who was

also unable to demonstrate a correlation. In order to understand this phenomenon a simplified pathway of carbohydrate utilization has been used as a guide. According to *Tasman* and *Brandwijk* (1937; 1938; 1940) glucose present in diphtheria cultures is rapidly converted into carboxylic acids (such as formic, acetic, propionic, lactic and succinic acids), and also ethanol is detectable. With maltose, acid formation is slower, the splitting of this disaccharide into glucose being the limiting step. When all the glucose has been consumed the pH rises again. The formation of acid is considered to be a truly fermentative dissimilation.

The simplified scheme is as following:

$$\text{Maltose} \xrightarrow[1]{\text{reaction}} \text{Glucose} \xrightarrow[2]{\text{reaction}} \text{Carboxylic acid} \xrightarrow[3]{\text{reaction}} CO_2 + H_2O$$

It is clear that the pH is influenced by the turnover rates of the reactions 1, 2 and 3. Reaction 2 is always the quickest one, so accumulation of carboxylic acids depends upon how rapidly glucose is supplied and how rapidly the carboxylic acids are oxidized. This latter step is dependent upon availability of free oxygen, and will be slower with a lower aeration rate. Thus the drop of pH with low aeration can be explained. Because growth rate is, unlike pH, unaffected by this low aeration level, some connection between carbohydrate dissimilation and toxin production seems probable. Addition of glucose instead of maltose leads within seconds to acidification. After the glucose addition is stopped, the pH goes down until all glucose is consumed, and then the pH immediately starts rising. When the glucose addition is monitored by a pH-controller, pH could be held constant within 0.05 units. Cultures thus controlled at pH 7.0 and 7.5 gave results comparable with a culture containing maltose under the same conditions, but without the pH-control.

In these experiments glucose consumption was about twice the amount of

Table 4-25 *Calculation of acid formation from glucose by* **C.diphtheriae**

Experiment D 90

–	pH Regulation by addition of alkali:	Total 3.1 grameq.
–	Constant addition of glucose:	Total 308 g
	Normal consumption of glucose:	Total 125 g
	Neutralized by alkali	183 g

Result: 1 grameq. per 59 g glucose
or about 3 acid groups formed from 1 glucose molecule

maltose in a parallel batch, i.e. the comparable amount.

Assuming glucose to be the limiting factor, we tried to increase the glucose consumption without influencing the pH-constancy. To achieve this, alkali has to be added as a pH-regulator in addition to a constant flow of glucose. In this way somewhat lower toxin yields have been obtained.

Although toxin production was low, this experiment enabled us to make a calculation concerning the amount of acid produced. Assuming that the glucose consumed with simple regulation (the first experiment, without alkali addition) is the maximum that can be oxidized further, the surplus in the second experiment is converted into acid only and is neutralized by alkali. On calculation, one gramequivalent of acid is found for every 59 gram of glucose (Table 4-25). Complete splitting into a carboxylic acid with 2 C-atoms (acetic acid), as for instance in **Clostridium thermoaceticum** (*Barker,* 1944; *Barker* and *Kamen,* 1945), could be an explanation.

The interest in the finding that low air flow rates result in low toxin titres was aroused again when the oxygen electrode became available. It appeared that only very low pO_2-values (below 3 mm Hg) impaired toxin formation. One is also referred to Fig. 4.21 where introduction of a mixture of equal

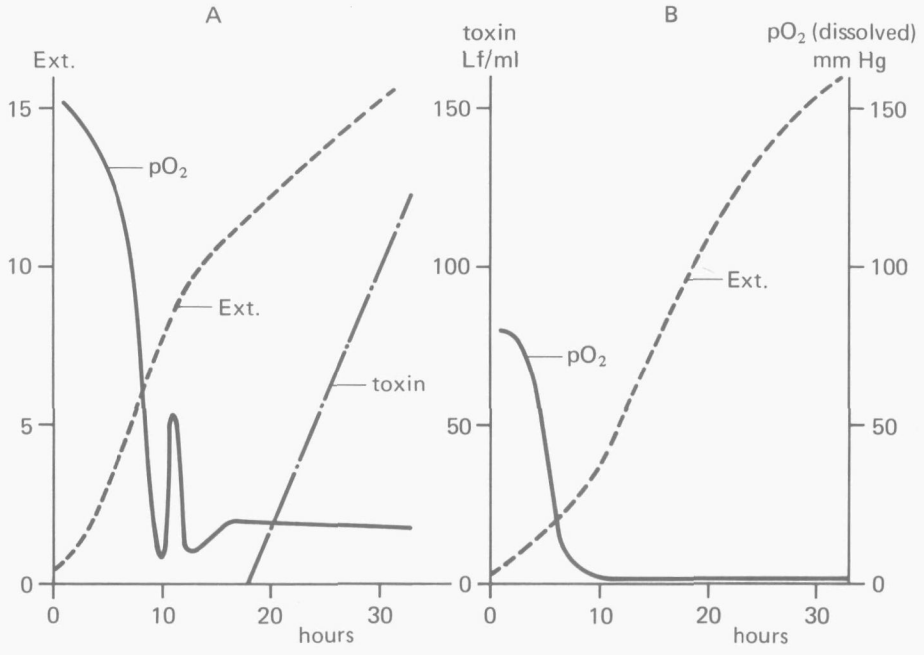


Fig. 4.21 Batch cultivation of **C.diphtheriae** in 7 litre culture. Flow rate 1 l/min; A: air; B: 50 % air/50 % nitrogen. Toxin is only produced in culture A.

Table 4-26 Comparison of two cultures of **C.diphtheriae**
Culture volume 7 l; toxin in Lf/ml after 50 h.
Maximal production rate in Lf/ml h.
DU 25: O_2-control at 15 mm Hg; pH-control with glucose at 7.8.
DU 26: no O_2-control; no pH-control.
Both cultures inoculated at the same moment with portions of the same seed culture.

Exp. no.	Toxin yield	Maximal prod.rate
DU 25	360	16.3
DU 26	160	2.9

volumes of air and nitrogen results in oxygen depletion of the culture and no toxin formation (right graph), while the use of air gives rise to a considerable amount of toxin (left graph).

Constant high yields (Table 4-26) have now been obtained in cultures in *Linggood* medium (Table 4-22) in which pO_2 is maintained at 15 mm Hg (10 % air saturation). In such an oxygen controlled culture, performed in *Linggood* medium with the normal amount of maltose (25 g/l) we found however, that in most cases, the pH-rise after the conventional drop (cf. Fig. 4.20) was greater than normal for cultures in which there is no pO_2-control. We concluded that rapid oxidation of acids formed from carbohydrates could be the cause. Therefore we started pH-control by the automatic addition of glucose (40 % solution) at the pH of normal levelling off (7.6-7.8).

CONTINUOUS CULTURE

The production of diphtheria toxin in continuous culture has been studied extensively in our laboratory. This is reflected in several publications (*Righelato* and *Van Hemert,* 1968; 1969; *Righelato,* 1969 a; 1969 b).

In experiments described above (p. 123) pH is kept at 7.0 c.q. 7.5 with glucose as the controlling agent (Fig. 4.22). In both cases the actual concentration of glucose in the culture is near zero, whereas growth rate and toxin production rate are relatively high. Growth was in fact limited by glucose, while good yields of toxin were obtained.

Growth limitation by glucose was applied to steady state continuous culture. In all continuous culture experiments described, the medium used was the same as before, with omission of maltose and pH was kept at 7.3 with HCl c.q. NaOH.

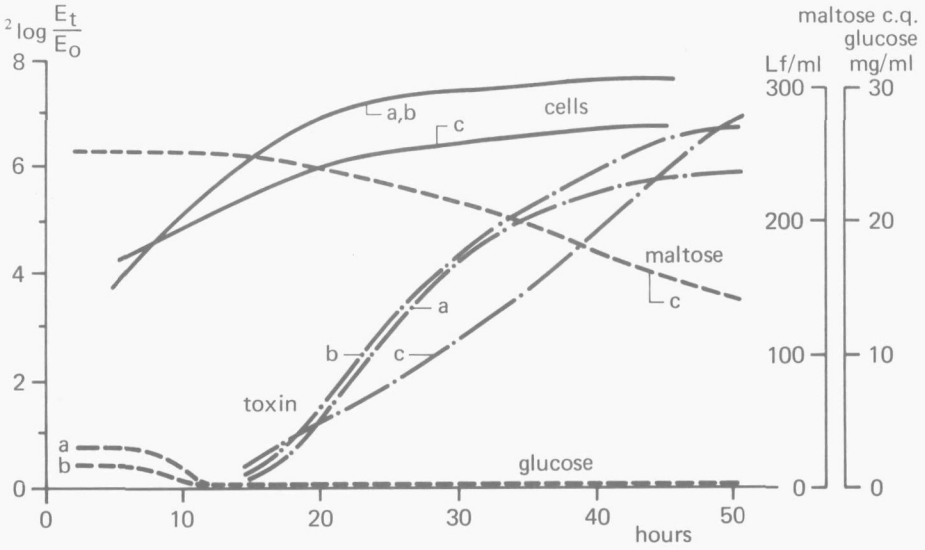


Fig. 4.22 Comparison of toxin production by **C.diphtheriae** in cultures with and without pH-control. 6.5 l modified Linggood-medium (prepared without maltose); 650 r.p.m.; air flow rate 10 l/min.
a: 0.25 % glucose added before inoculation;
pH-control at 7.5 with glucose.
b: 0.10 % glucose added before inoculation;
pH-control at 7.0 with glucose.
c: 2.5 % maltose added before inoculation;
no pH-control.

In Fig. 4.24 the dependence of the toxin concentration on the glucose concentration in the added medium is given. At a concentration of 0.14 M (25 g glucose per litre) a toxin titre of 200 Lf/ml is obtained, almost the same as the batch culture average titre. It can also be seen from the graph that the titre does not bear a strict relationship to cell concentration. Higher glucose concentrations have not been used, but it can be deduced from the figure that higher toxin yields may be obtained.

The relationship between toxin production and dilution rate is shown in Fig. 4.23, where toxin is given as protein (calculated on the basis of 1 Lf = 2.44 μg protein (cf. *Relyveld* et *al.,* 1964)) to show its connection with cellular and (total) extracellular protein. Toxin forms about 25 % of the extracellular protein at the higher dilution rates. The highest proportion of toxin to cell protein, about 10 %, was found at the growth rate 0.05 h^{-1}. This ratio was lower than that found during the maximum production phase

in batch cultures but it could be maintained for indefinite periods without fluctuation. The decrease in cell concentration at the lower dilution rates is due to two factors. There is a considerable amount of lysis at the lowest dilution rates, and the amount of glucose used to maintain the integrity of the viable cells, a process which does not contribute to growth, is proportionally greater when the cells have to be maintained in the culture vessel for a longer period of time, as is the case at the lower dilution rates.

We can conclude that at a dilution rate of 0.05 h^{-1}, i.e. a doubling time of 14 hours, stable and high toxin yields can be obtained in glucose limited chemostat culture.

The continuous cultures described so far have been performed at a pO_2 of 25 mm Hg, or about 15 % of air saturation. In Fig. 4.25 the influence of pO_2

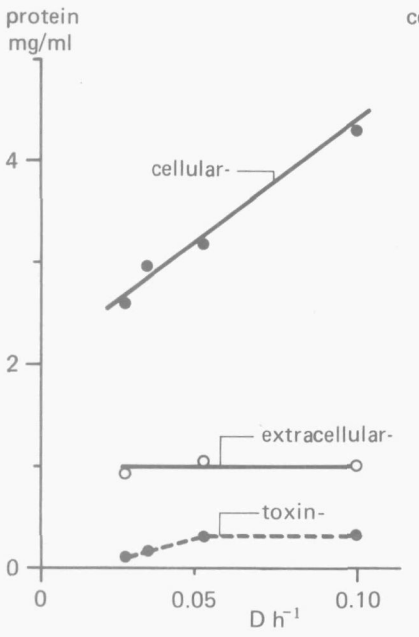


Fig. 4.23 Continuous cultivation of **C.diphtheriae.**
Influence of dilution rate on concentration of cellular, extracellular and toxin protein. Glucose concentration 0.085 M. pO_2 controlled at 25 mm Hg. Fe-concentration 6 μg at/l. pH controlled at 7.3.

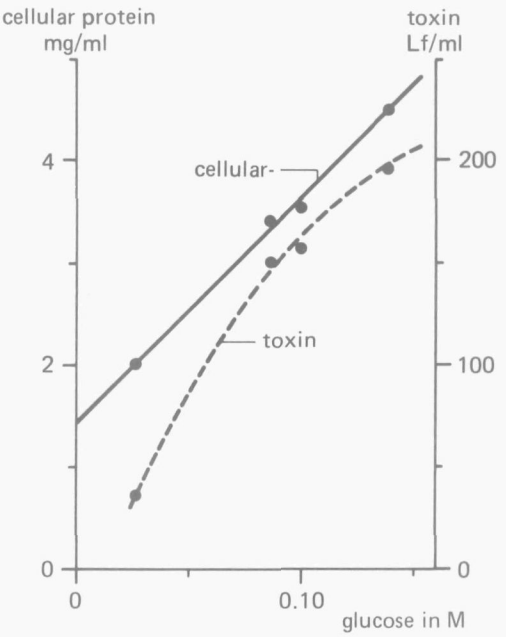


Fig. 4.24 Continuous cultivation of **C.diphtheriae.**
Influence of glucose concentration on concentrations of cell protein and toxin (Lf/ml).
Dilution rate D = 0.05 h^{-1}.

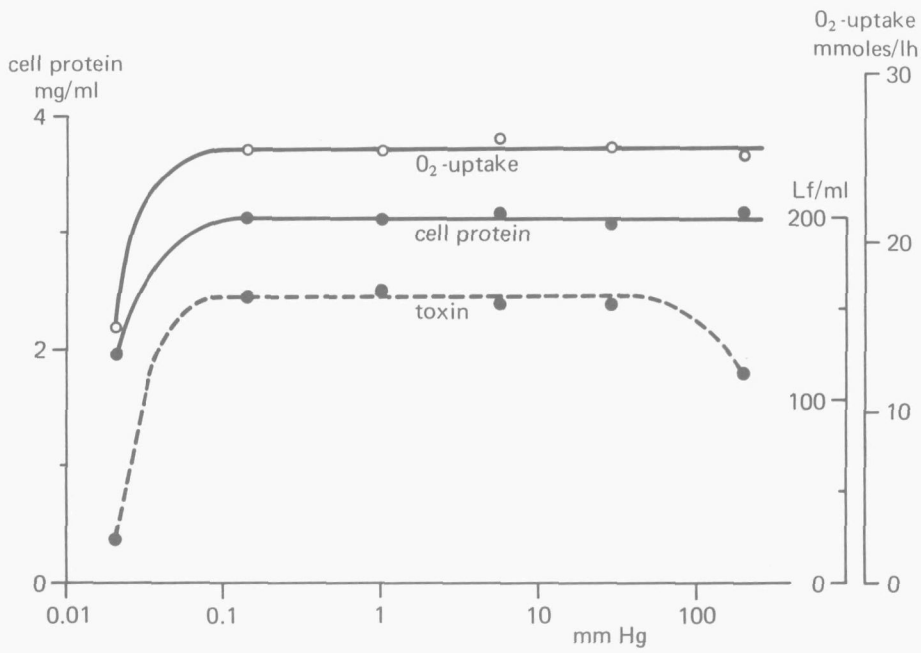


Fig. 4.25 Continuous culture of **C.diphtheriae.** Influence of dissolved oxygen tension (in mm Hg) on oxygen uptake and cell and toxin concentration.
D = 0.051 h^{-1}; glucose concentration 15.5 g/l;
Fe-concentration 5.0 μg at/l.

on toxin yield in continuous culture at a D = 0.051 h^{-1} is given. Over a wide range toxin and cell concentration are independent of pO_2. The oxygen uptake rate was 0.50 moles/l culture throughout, and the carbon dioxide was produced at 0.60 moles/l.

The observation that the pO_2 can be reduced to as low as about 0.2 mm Hg without affecting oxygen uptake is no doubt due to the degree of unsaturation of the terminal oxidative pathways, caused by limitation of growth by the major energy source, glucose.

At a pO_2 very close to zero (lower than 0.2 mm Hg but higher than zero) oxygen uptake was reduced and a partially fermentative metabolism ensued; acid(s) accumulated in the medium to a level of 0.20 M H^+ and only 0.30 moles CO_2/l was produced throughout, half as much as at higher oxygen partial pressures. The cell concentration was 40 % lower and the toxin titre 85 % lower.

If the 25 % reduction in toxin titre at 150 mm Hg oxygen is repro-

ducible, it might prove interesting to see what happens with the toxin titre at still higher pO_2-values.

In Fig. 4.25 a logarithmic pO_2 axis has been used to condense the scale. The control accuracy decreased from ± 1 % at $pO_2 > 100$ mm Hg to ± 10 % at 5 mm Hg. At exceedingly low oxygen concentrations (< 0.5 mm Hg) errors in the order of 100 % of the mean value might be present.

At the low pO_2-values, where toxin synthesis was inhibited to a great extent, some changes were observed in the iron containing enzymes present in the cell. These changes are of the same nature as those to be described below, and occur at iron concentrations inhibitory to toxin synthesis.

INFLUENCE OF IRON CONCENTRATION

The release of diphtheria toxin in the culture fluid is known to be inhibited by high iron concentrations in the medium (*Pappenheimer,* 1955; *Edwards* and *Seamer,* 1960). Although this finding has a firm basis, as will be shown, it led until recently to the belief that only aluminium vessels and stirrers could be used in diphtheria toxin production. In the actual situation in which concentrations in the order of 10 μM Fe are permissible, the dissolution of Fe ions from regular stainless steel may be disregarded.

In a series of preliminary experiments using batch culture it was shown

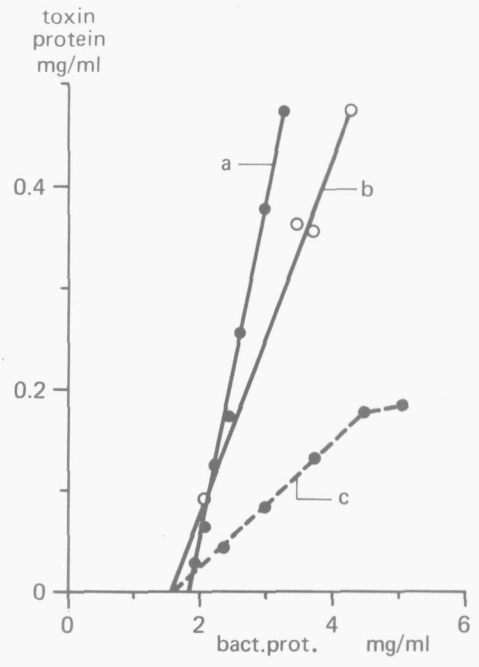


Fig. 4.26 The amount of toxin produced by **C.diphtheriae** at increasing bacterial concentrations in batch and continuous cultures.
a. Batch culture to which glucose was fed at growth-limiting rate.
b. Batch culture in which the glucose concentration was 5 g/l throughout.
c. Glucose-limited chemostat culture.
Each point represents a steady state obtained at a different concentration of glucose in the medium, and at a dilution rate of 0.051 h^{-1} (at D = 0.051 h^{-1} 9 g glucose gave 1 g bacterial protein; the medium without added glucose gave 1.5 g bacterial protein/l).

that addition of 5 to 10 μM Fe increased the toxin yield, whereas addition of 40 μM caused partial inhibition. The addition of 90 μM iron to a culture completely inhibited toxinogenesis, but did not influence the final concentration of bacterial cells; thus iron was not growth limiting. It is possible, however, that the decrease of the intracellular iron concentration below a critical value is necessary for toxin synthesis, as suggested by *Pappenheimer* (1955). If this is so, then toxin will not be synthesized until the cell concentration is high enough to lower the intracellular iron concentration to below the critical value. In Fig. 4.26 the toxin concentration is related to the cell concentration. In both glucose limited and excess glucose cultures no significant quantities of toxin were produced before the cell concentration rose above 1.6 g bacterial protein/l, at which time the iron content of the bacteria was 2.5 to 3.0 μg atom Fe/g bacterial protein.

In continuous culture, the intracellular iron concentration allowing initiation of toxin production, was in the same order (2.5-3.0 μg atom Fe/g bacterial protein) as in batch cultures. The concentration of several enzymes was determined in our laboratory by *Righelato* (*Righelato* and *Van Hemert*, 1969). He found that the concentration of intracellular non-heme iron and of three iron containing enzymes (expressed per unit of culture volume instead of per unit of cell mass) dit not vary with cell concentration (Table 4-27). Thus it would appear that the concentration of these enzymes was in fact limited by the low iron concentration.

As with batch cultures, the addition to the culture medium of 90 μg atom

Table 4-27 Continuous culture of **C.diphtheriae**
Relationship between bacterial concentration and some iron-containing enzymes.

Cell protein* g/l	Intracellular non-heme Fe μg atoms/l culture	Bacterial Cytochrome b extinction units/l culture	Succinate dehydrogenase mμ mole/ml/min	Catalase $k_{obs.sec^{-1}}$
2.0	4.1	8	-	-
3.30	3.0	9	140	10.4
3.85	4.0	10	190	12.2
4.50	3.3	8	140	12.2

* Cell protein concentration was varied by varying the concentration of glucose in the medium, 9 g glucose giving about 1 g bacterial protein.
The medium without added glucose gave 1.5 g bacterial protein/l.

Table 4-28 The effect of iron on the steady-state cell concentration and extracellular protein formation by **C.diphtheriae** in glucose-limited continuous culture.

Fe in medium μg atom/l	7	97
Cell protein, g/l	3.85	4.33
Non-toxin extracellular protein*, g/l	0.94	0.38
Toxin protein**, g/l	0.32	0
Cell non-heme Fe, μg atom/l	4.0	60
Cytochrome b, absorption units/l	10.8	40

* Total trichloroacetic acid precipitable protein in culture supernatant fluid less toxin protein.

** Calculated, assuming 1 Lf = 2.44 μg protein.

Fe/l completely inhibited toxin synthesis (Table 4-28). Immunodiffusion tests of supernatant fluids of the steady state culture with excess iron, and the steady state culture with iron restriction were made with a monospecific diphtherial antitoxic serum (kindly supplied by Professor *M.Raynaud;* Institut Pasteur, Paris) and a crude antidiphtherial serum which gave seven to eight precipitation lines with the culture supernatant fluid, the heaviest being the toxin. The toxin precipitation line could only be found in the restricted iron culture samples; the other lines were present in both restricted iron and excess iron samples. Antigens of the culture supernatant fluids, other than the toxin were also found but in higher concentrations in bacterial extracts. Their presence in the culture supernatant fluids may have been due to lysis of some of the bacteria. The higher concentration of non-toxic extracellular protein in the restricted iron cultures, described here and also observed by *Alouf* (1958) may reflect an increased tendency of the iron deficient bacteria to lyse.

Table 4-28 shows that iron addition, besides inhibiting toxin synthesis, brought about an increase in the concentration of diphtherial cytochrome b_{559}, the major heme containing enzyme of the PW 8 (PD) strain of **Corynebacterium diphtheriae** (*Pappenheimer* and *Hendee,* 1947).

The kinetics of disappearance of toxin from the culture indicate the rapidity with which toxin formation was inhibited. In steady state continuous culture, the formation of a bacterial product at a constant rate gives a constant concentration of the product (C_o) in the vessel. A step change may be applied to the culture, for example the addition of an inhibitor. The product present in the vessel before the application of the

change is then washed out exponentially by the continuous addition of fresh medium:

$$C_t = C_o e^{-D\,t}$$
$$\text{and } \ln C_t = -\,Dt + \ln C_o$$

where C_t is the product concentration t hours after imposition of the step change and D the dilution rate. If wash out occurs according to these kinetics, the time for the inhibitor to exert maximum inhibition will be small compared to the mean residence time (1/D) of the culture.

The disappearance of toxin from the continuous culture following the addition if iron is shown in Fig. 4.27 on a linear scale and plotted according to equation (2). The natural logarithm of the toxin concentration (C_t) falls linearly with a slope equal to - D and intercepts the ordinate at the zero time toxin concentration ($\ln C_o$). The inhibition of toxin synthesis thus was complete and immediate, to within the accuracy of measurement, upon the addition of the inhibitor.

DISCUSSION

The aspects of development of the production of diphtheria toxin studied comprised not only the medium but also the oxygen supply.

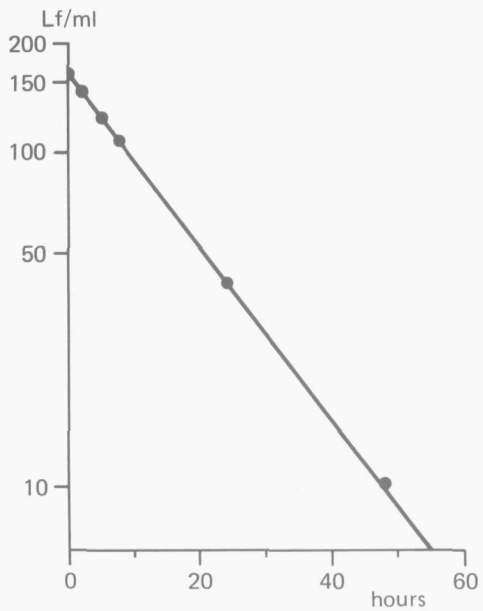


Fig. 4.27 The dilution out of toxin from glucose limited chemostat cultures of **C.diphtheriae** after inhibition of toxinogenesis by iron. At 0 hour the iron concentration in the vessel and in the medium was increased from 7 μM to 97 μM.
Dilution rate 0.051 h^{-1}.

The medium of *Linggood* and *Fenton* (1947) served as a basis. However, a study of papain digestion of the meat (beef instead of the original horse meat) indicated that a good digest was formed only if cysteine was added to reduce the enzyme maximally. The influence of the iron concentration has been studied extensively. For toxin production it was shown that the concentration normally present, 5-10 μM, is satisfactory. The inaccuracy and lack of reliability of the existing methods for the determination of iron at this level neither permit an accurate determination of the optimal iron concentration nor a check of this in every batch. Addition of extra iron quickly leads to inhibition of toxin production. It has been shown by continuous culture that, if this addition is performed during cultivation to a concentration of about 100 μM, inhibition is instantaneous and complete.

Cultivation at very low pO_2-values (below 0.2 mm Hg or approximately 0.1 % air saturation) gave low toxin yields; toxin production was decreased to a greater extent than cell production under these circumstances. These results were in accordance with the earlier observations (before the availability of the oxygen probe) that conditions of relatively low aeration gave rise to low toxin yields. There was also a difference in the course of pH during the cultivation compared with that of a good producing culture; due to a shift to a fermentative metabolism, more acids are formed and the pH goes down further. If no oxygen measurement or control is available, this course of pH is still the best check for suitable aeration conditions.

Where pO_2-control was available, setting at 15 mm Hg (10 % air saturation) gave excellent results. However, the reverse situation is observed in respect to carbohydrate metabolism; pH is rising quickly because of complete oxidation of the acids formed as intermediates. Additional pH-control with glucose as the correcting agent kept pH from rising uncontrollably.

Continuous culture with glucose as the limiting factor indicated that up to a concentration of 0.15 M in the added medium, the toxin yield increased with the concentration of glucose. A dilution rate of 0.05 h^{-1} was optimal for toxin production, on the basis of volume as well as cell protein. This holds true for all pO_2-values between 0.2 and 50 mm Hg (0.1 to 30 % air saturation).

The features of toxinogenesis in relation to the Fe content of the cells has been a matter of extensive investigation in our Institute. *Righelato* and *Van Hemert* (1968; 1969) found that toxinogenesis only occurred if the intracellular Fe concentration was below 2.5-3.0 μg atom per g bacterial protein. The existence of such a critical value was already suggested by *Pappenheimer* (1955). He also put forward the idea that the absence of toxin does not involve inhibition of synthesis of the toxin but incorporation of the protein into the cell in a non-toxic form. This may be realised in two ways,

namely by addition of an excess of Fe, or by reducing pO_2 to values at which partially fermentative catabolism occurred.

In both cases there was an increase in the concentration of heme containing enzymes and a decrease in the protein released from the cell. *Righelato* (1969 b) further studied the distribution of iron in the cells under both these extreme conditions and found that 2.8 % of the intracellular iron was present in the soluble fraction of bacteria disintegrated in the *Ribi*-press and the remainder bound on the respiratory particles. Under iron deficient conditions, which are optimal for toxin production, the Fe containing components of the respiratory particles were present in decreased quantities. Even after this study it is not completely clear whether decreased appearance of toxin is due to decreased formation p e r s e or to decreased release.

4.2.2 TETANUS TOXOID

INTRODUCTION

The disease caused by **Clostridium tetani** was known far back in history because of its characteristic symptoms; it was described already by *Hippocrates.* Its nature however remained obscure till *Carle* and *Rattone* (1884) demonstrated its transmissibility to animals, by injecting them with a suspension of a pustule of a tetanus patient. *Nicolaier* (1884; 1886) found that the inoculation of earth into mice and other animals led frequently to a disease closely resembling human tetanus. He found long bacilli in the pus at the site of inoculation, but was unable to bring them into pure culture. *Kitasato* (1889) finally demonstrated the aetiological role of the tetanus bacillus by isolating it in pure culture from pus and showing that such a culture contained a soluble toxin which could induce typical symptoms of tetanus.

The disease is characterized by muscular spasms, usually commencing in the neighbourhood of the site of infection. The infection itself is generally caused by contamination of a wound or raw surface. It is typical for injuries from war or traffic, and in new-borns by infection of the cut surface of the umbilical cord (tetanus neonatorum). In untreated persons the fatality may be very high (85 %). This is due to the fact that tetanus toxin is one of the most toxic compounds in nature. If a (non-immunized) person recovers from tetanus, the amount of toxin present in the body is evidently too low to induce antitoxic immunity as shown by *Tasman* (1959a; 1960).

As with diphtheria, the symptoms of the disease are due to a toxin produced by the microbe concerned. Tetanus toxin proved to be a good antigen, and antitoxic sera from animals have been prepared from the time of *von Behring* and *Kitasato* (1890), for therapeutic, and in a limited sense for prophylactic use.

Detoxification of tetanus toxin was first reported by *Descombey* (1924); he used formaldehyde, as first carried out for diphtheria toxin by *Glenny* et *al.* (1921).

The crude toxin solution is obtained from filtration of the culture fluid. In the regular production process at R.I.V., it is treated with formalin, and then purified by ultrafiltration and ammonium sulphate precipitation. Recently a new approach for purification as well as detoxification was brought under study, and will be mentioned at the end of this section.

Systematic vaccination with tetanus toxoid absorbed onto aluminium phosphate combined with diphtheria toxoid, pertussis and inactivated polio vaccine, has brought the fatality of this disease down to seven cases in 1970 in the Netherlands.

DEVELOPMENT OF CULTIVATION METHOD

In respect to method of cultivation **Cl.tetani** serves as the exception confirming the rule. The main difference with all the foregoing examples given is not the homogeneous cultivation that has been established here as well, but the cultivation vessel. A 200 litre stainless steel vessel, similar to the D-200 vessel, used for vaccine mixing was chosen. The following considerations influenced this choice:

1. **Cl.tetani,** unlike the other microorganisms studied, forms thermostable spores. This is the reason that the World Health Organization states in the manufacturing requirements for tetanus toxoid (*World Health Organization,* 1964): "All manufacturing processes up to the completion of detoxification shall take place in completely separate areas, using separate equipment". In such a one-purpose arrangement one large unit was preferred to the smaller 50 litre glass vessels.
2. As will be subsequently explained in more detail, homogenization of the culture is required, but without much inclusion of gas from the head space. This is distinct from the other processes described above, where rapid gas exchange with the head space was required and where vortex aeration was the best method. Mixing with a Vibromixer was indicated for tetanus toxin production.
3. Because of the requirement, mentioned in point 1, an independent unit for tetanus toxin production, at a certain distance from the rest of the laboratory, had to be created. Therefore it was built self supporting as well as compact. Preparation of the medium was projected to be performed in the stainless steel vessel and moreover, after completion of the cultivation, the vessel had to serve as a pressure container for Seitz filtration.

The earliest work on the subject in this laboratory was published in 1956 by *Sirks* and in 1962 by *Van Hemert* (1962b). Part of the later work to be described was carried out by *Van Wezel* (1967b) and is included with his permission.

The fact that **Cl.tetani** is an obligate anaerobe requires special precautions in cultivation. The medium of *Tarozzi* consisting of broth with meat cubes was widely used formerly. In the original version this medium was covered, after inoculation, with solid paraffin. Later it appeared that in rounded flasks, filled to the neck, anaerobiosis was guaranteed satisfactorily without paraffin. Yields were in the order of 1-3 Lf/ml.

In the classical studies of *Mueller* and *Miller* (1945; 1947; 1954) a medium was recommended that has maintained itself up to the present, although the cultivation method has been developed further. The composition of the medium appears in Table 4-29; the only modification compared with the

Table 4-29 Composition of medium for **Cl.tetani** per litre. Modified Mueller-Miller medium.

NZ-case, dependent on batch	15 to 25	g
Beef heart infusion	50	ml
Glucose	11	g
NaCl	2.5	g
Na_2HPO_4	2.0	g
KH_2PO_4	0.150	g
$MgSO_4.7H_2O$	0.150	g
$FeSO_4.7H_2O$	0.040	g
Cystine	0.25	g
Tyrosine	0.5	g
Uracil	2.5	mg
Ca-pantothenate	1.0	mg
Thiamin	0.25	mg
Riboflavin	0.25	mg
Pyridoxin	0.25	mg
Biotin	2.5	µg

5 N NaOH to pH 7.2

original formula is a different NZ-case concentration, and the use of $FeSO_4$ instead of Fe. It contains two "non-defined" components: a tryptic digest of casein (NZ-case) and beef heart infusion. *Latham* et *al.* (1962) claimed that they had adapted the highly toxinogenic strain of *Mueller* and *Miller* to growth and toxin production in the absence of beef heart infusion. We repeated *Latham's* experiments with the authentic strain. This strain indeed gave higher toxin yields in the infusion-free medium than our production strain. But in medium with beef heart infusion toxin yields were higher for both strains and did not differ much from one strain to the other. Moreover the acquired property of the *Latham* strain was easily lost by subculturing. We saw no advantage, especially in terms of consistency of production in using the adapted strain and the infusion-free medium.

The NZ-case is known to differ from batch to batch. It is probable that only amino-acids play a role. We analyzed the amino-acid content of the medium and the utilization during cultivation (Table 4-30). Six amino-acids are used up completely; leucine, phenyl-alanine and arginine are consumed in considerable quantity, whereas proline and valine increase in quantity during the later stages of cultivation, most probably in connection with lysis. Replacement of the NZ-case by the amino-acids utilized did not support growth.

Addition of an extra amount of the six amino-acids that are most rapidly

Table 4-30 Consumption of amino-acids in 120 l homogeneous culture of **Cl.tetani.** Only amino-acids that are completely consumed are given.
Toxin production is detectable at 48 hours and is complete at 150 hours.

cultiv. time hours	concentration in µmol/ml					
	serine	glutamine	glutamic acid	methionine	tyrosine	histidine
0	1.4	0.7	1.0	1.5	3.1	0.7
16	1.2	0	0	1.1	2.9	0
24	0.7	0	0	0	2.8	0
48	0	0	0	0	1.8	0
68	0	0	0	0	0	0

exhausted, maintaining the NZ-case, gave rise to earlier lysis of the cells and a lower toxin yield.

Mueller and *Miller* (1954) recommended cultivation in wide necked glass flasks for maximal toxin production. We confirmed this by comparing toxin production in an Erlenmeyer flask with a cotton wool plug, with that in a glass beaker covered with cotton wool between two layers of gauze. The latter gave consistently higher results, and it is not clear, whether this improvement is due to a better evolution of gases, or to a limited diffusion of air into the culture. In this connection attention may be called to the differences observed in the homogeneous culture flushed with air compared with flushing with nitrogen.

Before the development of the homogeneous cultivation method, production was performed in 20 litre quantities in 30 litre open cylindrical stainless steel vessels covered with a cotton wool layer between layers of gauze. Most of our experiments were done in 0.5 litre quantities in 1 litre glass beakers, covered in the same way.

Three series of experiments will be described here.

EXCRETION OF TOXIN

Unlike diphtheria toxin, of which in no stage of the culture process storage in the cell can be detected, tetanus toxin is present in the earlier growth phases in considerable quantities within the cell. *Raynaud* (1955) was aware of this, and assumed that the toxin molecule only leaves the cell after lysis, usually autolysis. *Raynaud* (1951) also suggested a method to cause forced lysis by keeping the cells in a hypertonic solution (originally 1 M NaCl and $^1/_{10}$ M sodium citrate; in our hands 1 M NaCl alone was as

suitable) for five days at 33°C (our modification four days at 4°C, followed by two days at 35°C).

This forced lysis has been used in two ways, one preparative and the other analytic. If a young culture of **Cl.tetani** ("young" to be defined as mainly consisting of un-lysed cells, containing a fair amount of intracellular toxin; v.i.; cf. the condition at day five in the right-hand graph of Fig. 4.28) are filtered and resuspended in a fraction (generally one fortieth) of the original volume of hypertonic solution, a concentrated toxin solution results. This solution, debatably called "autolysis toxin" is extremely suitable for hyperimmunization of horses used for antiserum production.

In order to compare intracellular with extracellular toxin concentration, the toxin extraction method described above was used to determine the quantity of toxin present in the cell. An aliquot from a 500 ml culture in a glass beaker, after homogenization, was used for this purpose. The determinations were carried out to find the optimal harvest time for either

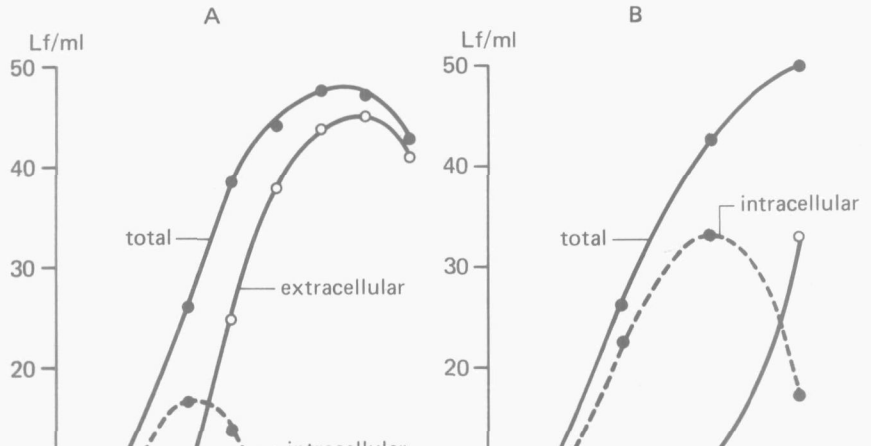


Fig. 4.28 Change of concentrations of intra- and extracellular toxin during cultivation of **Cl.tetani.** 500 ml culture. Total toxin calculated as sum of intra- and extracellular toxin.
A. Exp. VII. 9 serial transfers (see Table 4-31).
B. Exp. XIII. 66 serial transfers (see Table 4-31).

Table 4-31 Toxin production by **Cl.tetani** (500 ml culture)
Effect of seed culture on concentrations of intra- and extracellular toxin during cultivation.

Exp. no.	Maximum intracell. toxin		50/50 point	% after 5 days		Total Lf/ml after 3 days	Serial trans-fers
	days	Lf/ml		endo-	exo-		
VII	3.0	17	3.4	14	86	26	9
X	3.0	26	4.1	31	69	30	28
XII	4.5	30	5.7	63	37	31	42
XIII	5.0	32	6.3	77	23	26	66
XIV	4.5	30	5.5	59	41	26	78
XVII	3.8	30	4.6	33	67	26	138

the normal toxin production (maximum of extracellular toxin) or the preparation of "autolysis toxin" (maximum of intracellular toxin).

Fig. 4.28 gives the results of the determination of intra- and extracellular toxin, and the total toxin calculated from these two values. The two experiments differ only in respect to seed culture: in the case shown on the left-hand graph the culture was inoculated with the 9^{th} serial transfer of the seed culture on glucose broth, incubated under a H_2 atmosphere; in the case of the right-hand graph, the 66^{th} transfer was used. A gradual transition is observed; the excretion of toxin is slower in the first days in the latter culture, thus making it more suitable for obtaining cells with high toxin content for the preparation of "autolysis toxin".

Table 4-31 gives a further indication that the number of serial transfers influences the excretion pattern of the culture: up to the 66^{th} transfer the maximum of intracellular toxin comes later and is greater (column 2 and 3). With further transfer the maximum remains virtually constant but comes gradually earlier. The conclusion taken from these experiments is that it is advisable to check the excretion pattern of the seed culture from time to time in small experiments, especially when "autolysis toxin" is to be prepared. The exact nature of this rather uncontrolled change in behaviour has not been elucidated. It is at any rate connected with the ability of the cells to lyse. The easily induced changes in either direction give the impression that they are of a phenotypic character.

INFLUENCE OF STERILIZATION

Deterioration of a medium by oversterilization is a well known

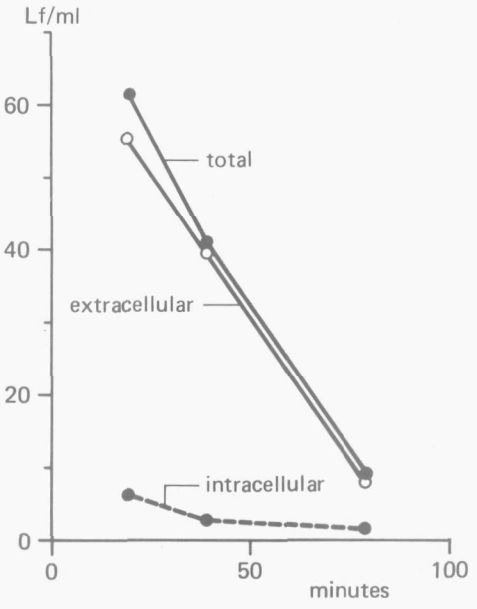


Fig. 4.29 Influence of sterilization time of medium (in minutes at 120°C) on toxin production (after 7 days cultivation) by **Cl.tetani.**

phenomenon. As Fig. 4.29 shows, this is also true for tetanus toxin production in the medium of *Mueller* and *Miller.* If one compares, however, the results after sterilization for 20 minutes at 120°C with those after a "Koch-treatment" (two times 100°C for 30 minutes), the former treatment gives a 50 % higher toxin yield. The change in the medium, responsible for this improvement, is unknown.

OPTIMAL CONCENTRATION OF CASEIN DIGEST

The influence of the batch of NZ-case on toxin production has been mentioned before (p. 137). It was necessary not only to check samples of the batches but also to determine the optimal concentration. This appeared to lie, dependent upon the batch used, between 15 and 25 g/l. In order to further optimize the conditions for toxin production, once a batch of NZ-case was chosen and the approximate optimal concentration determined, the method of *Box* (1957) was applied. This method called EVOP (Evolutionary Operation) consists of slightly varying the optimal value of the parameters, not in the experiments but in the actual production process. It is necessary that the variations are chosen such that the yields are in all cases acceptable. An example of the application of this EVOP method is illustrated in Table 4-32. Three concentrations of the casein digest and two incubation times were combined, giving six sets of conditions. Each set was

Table 4-32 Toxin production by **Cl.tetani.**
Application of EVOP-method for optimalization of process.
Results from three cycles of six batches each (except last column).

Average of batches with	Lf/ml		Lf/mg N in toxoid over 1 cycle
	toxin	toxoid	
7 days incubation	52.2	43.8	801
8 days incubation	47.7	42.3	765
17.5 g/l NZ-Case	55.0	46.7	885
20.0 g/l NZ-Case	49.3	41.7	802
22.5 g/l NZ-Case	45.8	40.8	663

used three times, giving a total of 18 batches. The relative accuracy of the *Ramon* flocculation test is supposed to be in the order of ± 10 %. So there is a significant difference in the toxin values shown in the table, the average of nine figures for incubation time and six for concentration of casein digest. The best conditions in this scheme proved to be seven days incubation at a NZ-case concentration of 17.5 g/l.

DEVELOPMENT OF HOMOGENEOUS CULTURE

In some preliminary experiments in a 5 litre "Bilthoven Unit" fermentor a reasonable toxin yield was obtained. To reproduce the situation existing in the wide, cotton wool covered containers, air was blown over the culture; stirring was kept low so as to ensure good dispersion of the cells without excess aeration, as this would impair anaerobiosis and stop the growth of the culture.

Further experiments were performed in vessels with Vibromixers. This type of mixing has the advantage of high mixing capacity without formation of a vortex. After extensive experimentation in transport vessels (C-containers, cf. p. 32) the D-200 vessel was chosen for routine production. The following aspects called our special attention:

Air and Nitrogen

Repeated experiments have shown that the effect of air is more than stripping of gases: if nitrogen is used instead of air **Cl.tetani** grows equally well but the toxin yield is lower. If the curves for total toxin concentration in Figures 4.30 and 4.31 are compared, the most striking observation is that, although nitrogen gives somewhat higher values for the intracellular toxin

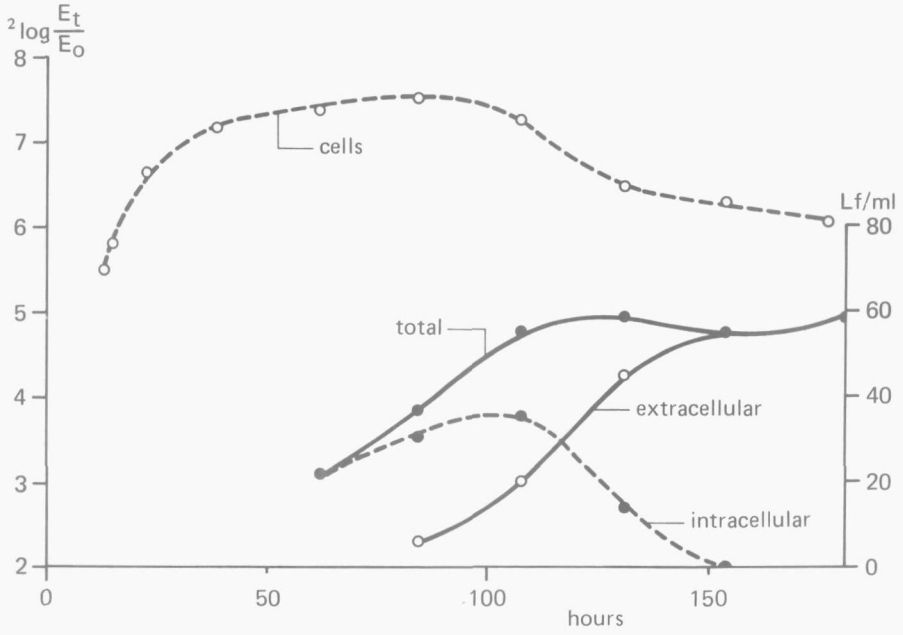


Fig. 4.30 Change of intra- and extracellular toxin concentration in 120 litre homogeneous culture of **Cl.tetani,** flushed with air, except for the first 24 hours, during which N_2 is used. Total toxin calculated as sum of intra- and extracellular toxin.

level, the total toxin yield, after most of the toxin has been excreted, is appreciably lower (*Van Wezel,* 1967b). In other words, in a nitrogen atmosphere, part of the toxin formed is easily decomposed at the end of the cultivation. Partial inhibition of proteolytic enzymes set free after lysis of the cells by the higher redox potential in air, might be an explanation. Therefore flushing with air is recommended. Because initiation of growth is impaired by oxygen, it is advisable to use nitrogen during the first 24 hours, and air thereafter. Table 4-33 gives a comparison of the average yield, using the "old" method in 20 litre vessels without agitation, with the yield using the new "homogeneous" method. The yields are given as Lf/ml of the toxoid, not of toxin; it is in the form of toxoid that the product is further processed. It is deduced from the table that the homogeneous cultivation method gives a 50 % higher yield.

Incinerator

There are two reasons for paying attention to the effluent gas from the

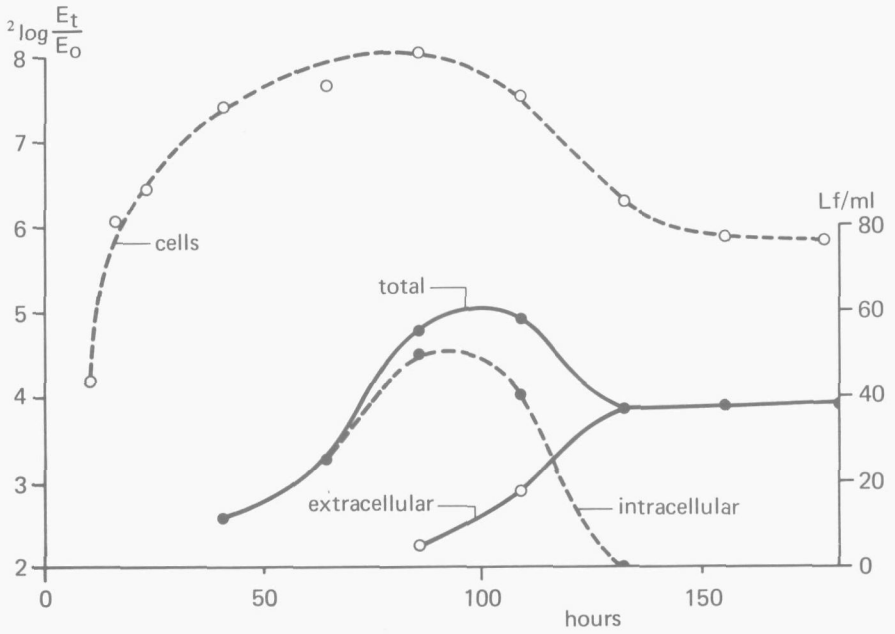


Fig. 4.31 Change of intra- and extracellular toxin concentration in 120 litre homogeneous culture of **Cl.tetani,** flushed with nitrogen.
Total toxin calculated as sum of intra- and extracellular toxin.

Table 4-33 Average tetanus toxoid titres in pools in the routine production at R.I.V.; static compared with homogeneous culture.

type of culture	litres produced	number of pools	titre Lf/ml		
			average	lowest	highest
static	5446	13	38.9	32	47
homoge-neous	2549	9	58.5	44	66

tetanus culture. First there is the possibility that germs escape from the culture with the gas; second, the specific and unpleasant odour of the effluent gas might become a serious problem for the environment with a 120 litre culture. The gas may be washed through 5 N sodium hydroxide, a good solution for both decontamination and deodorization, although only practicable for a small (experimental) culture. But for the flow rates used in

large cultures (25-50 l/min) an incinerator was developed instead, which proved to be completely satisfactory.

The principal arrangement of this incinerator is shown in Fig. 4.32. Gases are injected in the central tube directly against an electric heating element; they are led back through a concentric outer tube filled with chips of stainless steel. The total internal volume is 3.3 litre. With a flow rate of 25 l/min this gives a residence time of eight seconds at room temperature, but of only about four seconds at 300°C.

In Table 4-34 the sterilizing effect of the incinerator is tested with an aerosol of **Bacillus stearothermophilus,** made in a *Collison* spray (kindly made available by *Dr.Bartlema* of RVO-TNO). At each setpoint temperature, 115 litres air were sent through the incinerator, with 400-1100 spores per

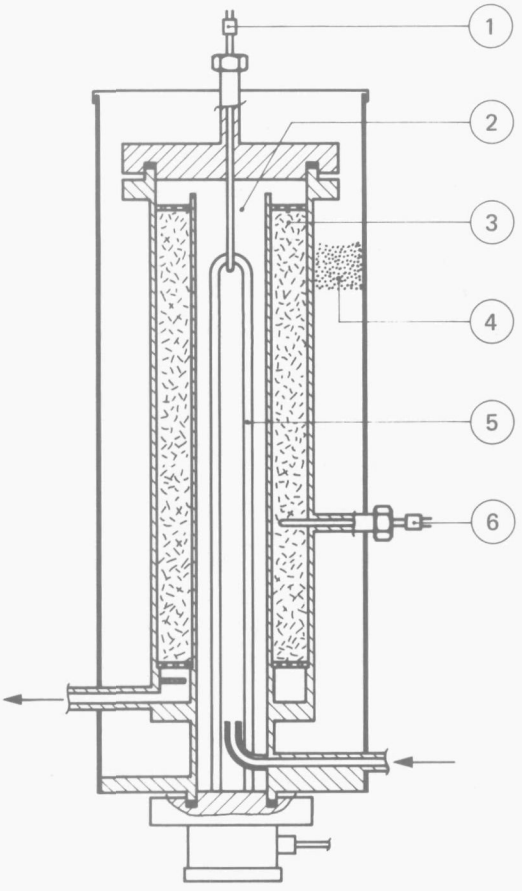


Fig. 4.32 Incinerator for exhaust line of **Cl.tetani** cultivation (schematic).
1. "Control"-thermocouple. 2. Inner reaction chamber. 3. Outer reaction chamber with stainless steel shavings. 4. Thermal insulation. 5. Heating element. 6. "Safety"-thermocouple.

Table 4-34 Penetration percentages in incinerator at different setpoint temperatures. "Safety" temperatures, measured at point 6 (see Fig. 4.32), serve as approximations of reaction temperature.
Air flow rate 25 l/min at room temperature. Data on aerosol are mentioned in the text.

Temperature		Penetration
setpoint	safety	%
400	275	< 0.002
300	210	< 0.002
250	175	< 0.002
200	135	1
150	107	34
20	20	35

litre. The net recovery without heating is 35 %. At all setpoint temperatures (measured with "control" thermocouple (1), in Fig. 4.23) from 250°C onwards no germs are retrieved, indicating a survival of less than 0.002 %. The temperature at the "safety" thermocouple ((6) in Fig. 4.32), which is placed a little over halfway down the stream path, is then 170-180°C. In order to achieve a fair safety margin the temperature at this point should be kept at 300°C. This implies a setpoint temperature for the heater of 500-600°C.

The above mentioned results are roughly in accordance with the data given by *Elsworth* et *al.* (1961). They find, with exposure times in the order of 1-2 seconds, penetration percentages decreasing from 0.1 to less than 1.8×10^{-7} (most probably between 10^{-10} and 10^{-11}) with temperatures rising from 210 to 330°C. Even when taking into account that exposure times in our case are double that of *Elsworth* et *al.* (loc.cit.) and that the apparatuses used are not completely comparable, it is undoubtedly a safe conclusion that a reaction temperature of 300°C gives complete killing of the few germs to be expected.

An extra safety arrangement is brought into action in case of failure of the incinerator. See Fig. 4.33. As soon as the temperature at the "safety" thermocouple comes below a predetermined value (say 300°C), two electro-valves, one at the gas entrance and one at the gas exit line of the fermentor, are closed.

In Fig. 4.34 a picture is given of the D-200 vessel as used for tetanus toxin production. The panel is of the same outer form as the one described in

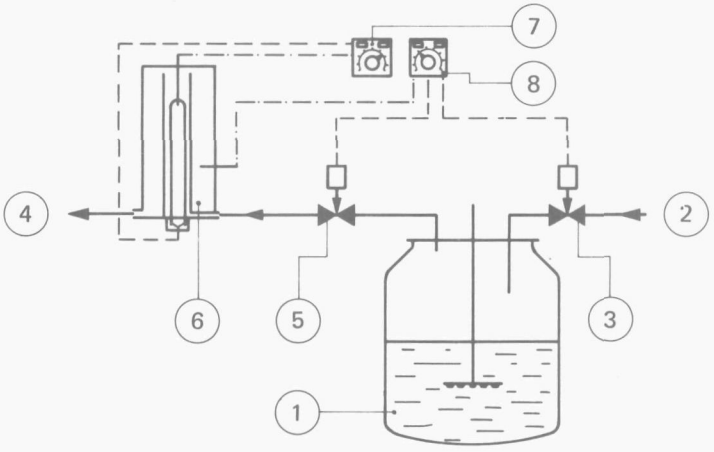


Fig. 4.33 Schematic representation of setpoint and safety circuit of incinerator (see also Fig. 4.32).
1. Fermentor. 2. Incoming gas. 3. Solenoid valve on incoming gas. 4. Outgoing gas. 5. Solenoid valve on outgoing gas. 6. Incinerator. 7. "Control"-thermostat. 8. "Safety"-thermostat.

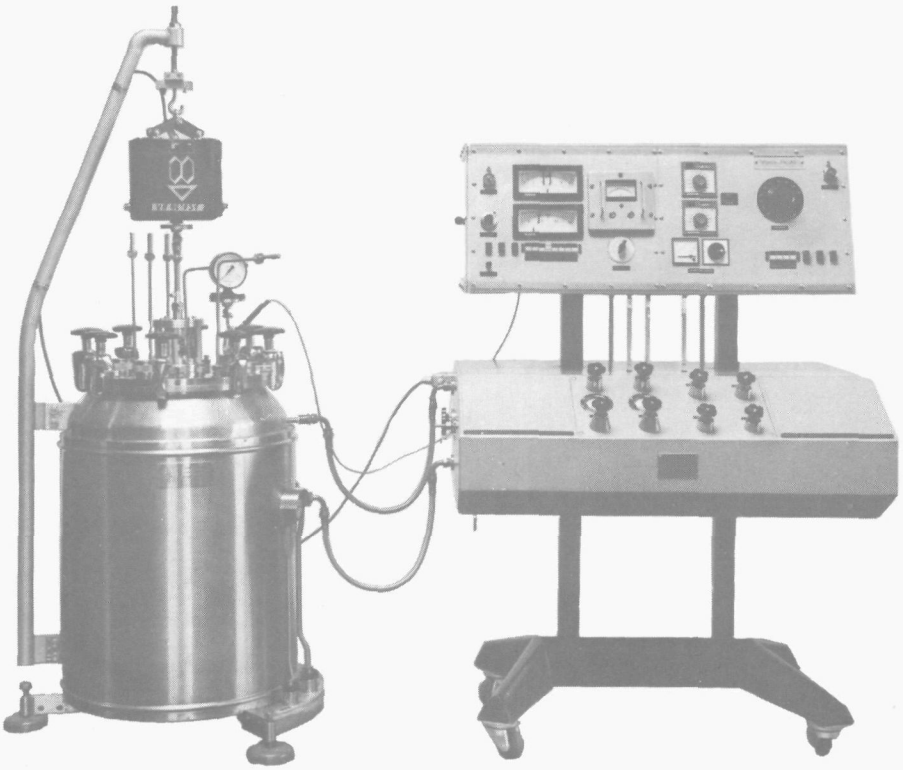
Chapter 3 (p. 61) as "Paljas Unit". In this version the incinerator is built in and placed in horizontal position in the left-hand part of the lower body. The controls for heating and safety of this incinerator are visible in the upper right part of the panel.

DISCUSSION

Among the microorganisms to which the concept of unit process in vaccine production has been applied, **Cl.tetani** takes a special position. It is an anaerobic spore-former whose thermostable spores have led to the W.H.O. requirement that tetanus toxin should be prepared in a separate confined space. It led us to the conception of a different type of culture vessel than the one used for all other microorganisms studied.

From the technological point of view it led to one of the most elegant of the processes devised, with preparation of the medium, sterilization and cultivation in one and the same vessel and sterile filtration using this vessel as the pressure container. The fact that also a satisfactory solution was found for safe disposal of the spent gas, and at the same time for deodorization, adds to the character of self-contained unit, functioning smoothly in a limited space.

An unsatisfactory aspect of the present way of cultivation is the fact that



(Courtesy N.V. Apparatenfabriek van Doorn, de Bilt)

Fig. 4.34. *"Paljas-T" unit with built-in incinerator. 125 litre vessel for cultivation of* **Cl.tetani**

the medium still contains two undefined components: beef heart infusion and a tryptic digest of casein. Although *Latham* developed a strain for which he claimed good toxin production in the absence of the former component, we found this strain rather unstable, and moreover giving higher toxin yields with beef heart infusion than without. *Mueller* and *Miller* who developed a strain and medium such as we used, were not able to define the essential components in the NZ-case, and neither did we succeed in this problem.

The delayed excretion of the toxin from the cells formed an intriguing point of investigation. The only parameter so far found to somewhat influence this phenomenon is the serial transfer of the seed culture in glucose broth. Early lysing cultures give less accumulation of toxin in the cell, although this cannot explain entirely the difference between cultures having

70 % of the final total toxin production contained in the cell at some moment, and those with no more than 30 %. It is noteworthy in this respect that we have found repeatedly (cf. Fig. 4.30 and 4.31) that in homogeneous cultures flushed with nitrogen more toxin is accumulated in the cell than when flushing with air.

In establishing a homogeneous culture it was necessary to effect good mixing of the fluid without too much mixing with the gas in the headspace, because it was found that air in the headspace gives a higher toxin yield: too much aeration would impair growth. There is no explanation of the "air effect". Probably toxin is degraded quicker in nitrogen atmosphere, perhaps by the action of proteolytic enzymes inactivated at higher redox potential values. If degradation of the toxin is the cause, this could be connected with the finding of *Peetoom* and *Van der Veer* (1967), who observed that fresh tetanus toxin and tetanus toxoid carry two antigenic determinants which elicit antitoxin (called 1 and 2). Determinant 1 is lost by degradation, occurring spontaneously or by treatments such as freezing. Such degradation may lead to a lower flocculation value with the *Ramon* test.

4.2.3 STAPHYLOCOCCUS α-TOXOID

INTRODUCTION

The application of staphylococcus α-toxoid as a prevention against infections caused by **Staphylococcus aureus** is rather limited. The production method was quite considerably developed by us and in many respects is a good example of the advantage of homogeneous cultivation technique.

St.aureus is the causative organism for a number of suppurative processes in man such as osteomyelitis, pyaemia and mastitis, and skin infection as boils and carbuncles. Wide and sometimes unjudicious use of antibiotic therapy has increased the number of antibiotic resistant staphylococci. Hence it forms a special threat in hospitals.

Although observance of personal hygiene, and the use of aseptic techniques in nurseries and medical and surgical wards are the best available preventive measures, the use of staphylococcus α-toxoid, especially against furunculosis and mastitis is still recommended by some investigators.

St.aureus produces an exceptionally great amount of extracellular products of high molecular weight. Four toxins, all with hemolytic action and hence also called hemolysins have been identified: α-, β-, γ- and δ-toxin. Because most pathogenic strains contain α-toxin (and also coagulase according to *Gladstone* and *Glencross,* 1960), and staphylococcal infections give rise to increased anti α-antitoxic antibody levels (*Tasman* et *al.,* 1959b) it is considered as the most important toxic product.

Certain importance also has been ascribed to leucocidins, mainly to the so-called Panton-Valentine leucocidin (*Bänffer,* 1961). The importance of this compound (or probably group of compounds, cf. *Woodin,* 1959; 1960) is still not clear and it has, as far as known never led to a prophylactic preparation.

The α-toxin is purified, detoxified with formalin, and injected with aluminium phosphate as an adjuvant.

For all experiments and for production strain Wood 46 (NCTC 7121) was used.

DEVELOPMENT OF CULTIVATION METHOD

At the onset of our experiments, the culture method used was a modification (*Tasman* and *Van der Slot,* 1953) of the original method of *Parish* and *Clark* (1931). In this method *St.aureus* was grown on a soft agar medium, containing a pancreatic digest of horse meat, peptone and mineral salts. Cultivation was performed in hermetically closed Roux bottles. Part of the air on top of the medium was taken away by suction and replaced by carbon dioxide to 30 volume %, by means of a fairly complicated procedure.

The use of carbon dioxide as a means to promote α-toxin formation in **St.aureus** is suggested by several authors. *Parker* et *al.* (1925; 1926) recommended already the use of this gas. *Burnett* (1930) and also *Gladstone* (1938) considered, under their circumstances 20 % carbon dioxide and 80 % air or oxygen as optimal. *Leonard* and *Holm* (1935) found a mixture of 80 % carbon dioxide and 20 % oxygen most favorable; they used closed tanks for cultivation. *Casman* (1940) found no toxin formation when bubbling air through the culture fluid, a low toxin production when using oxygen, and a maximal one when bubbling a mixture of 80 % oxygen and 20 % carbon dioxide.

The medium used by us was the same as for production of hyaluronidase by *St.aureus* (*Van Hemert,* 1958). The composition is given in Table 4-35.

In a set of experiments in 7 litre culture shown in Table 4-36 the

Table 4-35 Composition of culture medium for α-toxin production by **St.aureus,** in g/l.

Yeast-extract (Gistex, Delft)	20
Proteose peptone (Difco)	5.0
NaCl	2.5
Na_2HPO_4	11.6
KH_2PO_4	6.0
glycerol	5.0

pH 7.2

Table 4-36 α-toxin production by **St.aureus,** strain Wood 46, in different gas atmospheres.
Flowrate 4 l/min.; stirrer speed 500 r.p.m.

Exp. no.	gasmixture, vol. %			α-toxin Lh 10/ml
	air	CO_2	O_2	
42	100	-	-	71
43	85	15	-	125
44	75	25	-	250
45	100	-	-	50
46	60	40	-	83
47	-	40	60	333
48	100	-	-	83
49	-	25	75	333
50	-	75	25	333

composition of the gas mixture blown over the culture is varied (*Van Wezel* and *Van Hemert,* 1965). Toxin yields are expressed as L_h 10/ml.

The L_h 10 is determined by bringing together increasing volumes of culture fluid with 0.1 antitoxic unit α-antitoxin and rabbit erythrocytes. The volume in ml just not giving hemolysis is the L_h 10. With volumes smaller than 1 ml, the reciprocal value (L_h 10 per ml) is commonly used. This test is used instead of the *Ramon* flocculation test, because the antisera at our disposal gave no consistent flocculation.

The results listed in Table 4-36 were obtained in three groups of three experiments. One of each group served as a control in which only air was used. The results show that oxygen/carbon dioxide mixtures of 25/75, 60/40 and 75/25 give comparable yields of toxin. Among the air/carbon dioxide mixtures 75/25 is the most favorable one. A minimum of 25 % carbon dioxide is apparently needed for satisfactory toxin production. If, in an air/carbon dioxide mixture, the carbon dioxide concentration is increased above 25 %, the toxin yield is lower, most probably because not enough oxygen (only 12 %) is present. So we suppose that a minimum of 15 % oxygen (as pure oxygen or in air) is needed for optimal α-toxin production.

With a mixture of 75 % carbon dioxide and 25 % oxygen consistently good results have been obtained on 40 litre scale, with toxin yields of 333-400 L_h 10/ml.

In Fig. 4.35 a curve is given, showing the growth and toxin production. It is only near the end of the logarithmic phase that appreciable quantities of α-toxin appear (the first point between parentheses is most probably lower than stated !). The reason for this late appearance has not been studied.

DISCUSSION

The suitable gas mixture for promotion of α-toxin production by **St.aureus** has called the attention of several investigators between 1925 and 1940. *Casman* (1940) was the first to realize that the effect of the gas atmosphere in a closed system, as was almost invariably used, depends upon the ratio of culture volume, culture surface (with solid media) and volume of headspace.

In our experiments in homogeneous culture it appeared that the influence of oxygen and carbon dioxide concentrations are independent of each other; most probably the oxygen concentration is in many cases the growth rate limiting factor, and growth rate could influence α-toxin production rate (v.i.). The effect of carbon dioxide is not yet known, but a minimum of 25 volume % of this gas seems to be required for maximal toxinogenesis.

The rate of toxin production in relation to the growth rate may be considered on reference to Fig. 4.35. α-toxin is first detected in the culture fluid towards to end of the logarithmic growth phase. We have not been able to detect accumulation of α-toxin in the bacterial cell, which is in

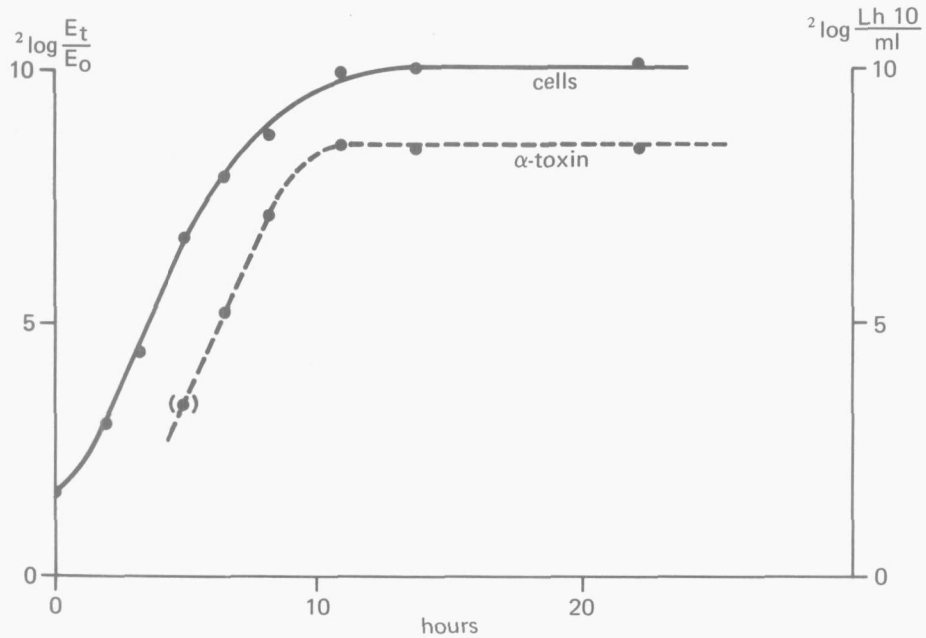


Fig. 4.35 Growth and α-toxin production by **St.aureus,** strain Wood 46. Experiment 50 (see also Table 4-36).
Both parameters expressed in ^{2}log for reasons of comparison.

accordance with the findings of *Raynaud* et *al.* (1955). Toxinogenesis seems to start at the relatively late growth phase mentioned. It is also notable that during a couple of hours (in Fig. 4.35 between five and ten hours after inoculation) the rate at which α-toxin is formed is appreciably higher than the growth rate. A similar phenomenon is observed in the formation of the enzyme hyaluronidase by **St.aureus** (*Van Hemert,* unpublished data, 1957; *Van Hemert,* 1958). In this case the production of the enzyme is maximal only if the growth rate is submaximal, such as by oxygen limitation. Under these conditions, the rate of production of hyaluronidase is, during a certain few hours, higher than growth rate. *Rogers* (1954; 1956) also found during hyaluronidase production by **St.aureus,** that this enzyme appears rather late in the growth cycle, but is then subsequently produced thereafter at a rate higher than growth rate. Neither the formation of α-aminobutyric acid acting as an inhibitor, nor extra need for thiamine, as suggested by *Rogers,* gives a good explanation.

The α-toxin produced in 40 litre culture with a gas mixture of 75 % carbon dioxide and 25 % oxygen is first purified and then detoxified. Details on the procedure for purification and detoxification are given by *Van Wezel* and *Van Hemert* (1965).

4.3 Viral Vaccines

The multiplication of viruses, needed for the preparation of viral vaccines for human application, whether live of killed, is in most cases accomplished in in-vitro cultures of animal tissue cells. These cultures are normally derived directly from animal organs, such as monkey kidney, and the cells are dispersed by the use of trypsin. The cultivation is performed almost exclusively in a monolayer, i.e. one (sometimes more) layers of cells attached rather firmly to the inner wall of a glass vessel, which spread until the whole surface is covered. This method was brought into practice, mainly by the work of *Earle* et *al.* (1954).

The monolayer serves as a substrate for virus multiplication. Despite extensive work on cell cultivation over the past decade, the special conditions for virus production have received little attention. Animal viruses

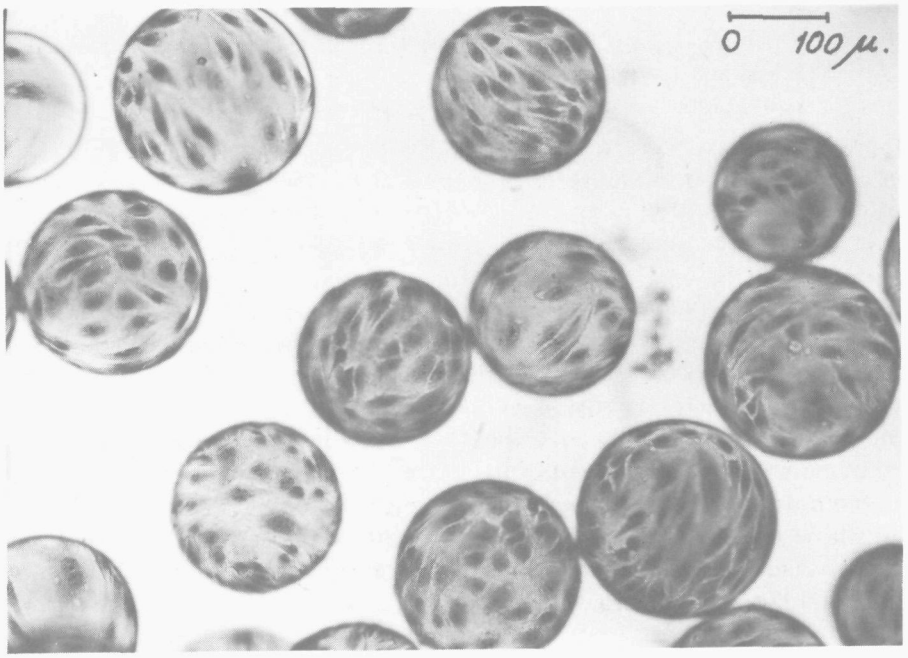


Fig. 4.36. Growth of euploid rabbit skin cells on DEAE-Sephadex beads. Outgrown culture. (*van Wezel*)

represent at most about 5 percent of the dry weight of their hosts and hence would not appear to overload the nutritional demand of a cell. For monolayer cultures at least, there may be no demand for further information on virus cultivation conditions if it is tacitly assumed that the conditions most favorable for cell multiplication are also optimal for virus replication.

Homogeneous culture of tissue cells and propagation of viruses on these cells in the same system has great advantages. It is possible to avoid the high risks of contamination inherent when using monolayer cultures which must be opened repeatedly for inoculation, refreshment of medium, and harvesting. In homogeneous culture one vessel replaces a multiplicity of small bottles and hence the few required operations can be performed using elaborate precautions that could not be applied economically to monolayer cultures. Furthermore, in homogeneous cultures, the possibility to introduce control such as for pH and oxygen tension obviates the need for medium refreshment that is standard practice in monolayer culture.

Homogeneous culture of tissue cells, however, also presents specific problems. It is often difficult to obtain and to keep a suspension of single cells. In most cases animal cells will not multiply in free suspension, but require attachment to a solid surface. Certain cell-lines have lost this requirement and can be grown in suspension. These cells can be subcultured indefinitely but have an abnormal karyotype; in this, and in other respects they resemble cancer cells.

The BHK cell-line selected by *Macpherson* and *Stoker* (1962) is a well known example and has been extensively used as a virus host, e.g. for foot and mouth disease virus (*Capstick* et *al.,* 1962), Semliki Forest virus (*Telling* et *al.,* 1967) and Rubella virus (*Vaheri* et *al.,* 1965). HeLa cells have also been frequently used, even for steady state continuous cultivation of viruses, as described by *Gori* (1965) for polio virus.

Strict regulations exist for production and control of viral products for human injection, and the cell-lines are banned for this purpose because of the supposed risk of oncogenicity (cf. *Van Hemert,* 1964c). Only primary cells, i.e. cells taken directly from normal tissue and subcultured only once, are accepted. These cells have the chromosomal constitution of the tissue from which they have been taken.

Hayflick (1961; 1967) developed cell-lines from human embryonic tissue which he called cell strains, and which maintain their diploid character after repeated subculture, while exhibiting a finite lifespan of about 50 doublings. *Hayflick* argues that these diploid strains are at least as safe as primary cells for use in human vaccine production and in some respects may be superior since they can be tested extensively for contaminant viruses before use. Moreover, the diploid cell strains improve the standardization of the vaccine production process, because seed culture can be stored at low temperature.

Diploid cells would seem to be the perfect host, since they allow the propagation of almost any human virus; they can be screened for later virus long before use and they attach easily to the DEAE-Sephadex beads (v.i.) because these are free from mechanical imperfections. In practice, however, these cells will require more generations in vitro than primary cells and it is felt that this may introduce some risk.

Primary cells and diploid cell strains cannot at present be cultivated in free suspension. This difficulty may be circumvented by the use of the "microcarrier" method of *Van Wezel* (1967a), in which the cells adhere to DEAE-Sephadex beads (grade A 50, about 100 μ diameter) and grow to form a confluent monolayer over the surface of the bead (Fig. 4.36). In a suspension such as this, the environment of each cell is almost identical and the culture can be termed quasi-homogeneous.

The way now lies open for the further development of the homogeneous culture method using "microcarriers". In case euploid cell-lines are allowed, culture is easier than with primary cells. In the latter case the current trypsinization methods, giving much cell debris, need to be improved considerably.

An other point of special study is the step of virus propagation. There is a difference in approach depending upon whether the infected cells die or continue to replicate, establishing a situation that is labeled carrier state or persistent infection or concurrent growth. Polio infection of monkey kidney cells illustrates one extreme in which cell metabolism is rapidly disrupted and the cell destroyed. In such a case it is necessary to infect an outgrown cell culture. At the other extreme, the infection of BHK-cells with Rubella virus apparently causes little change in cellular metabolism except that virus is continually produced. One can even store the Rubella infected cells in the frozen state and resume cultivation after thawing. With this type of persistent infection one can proceed for a long time harvesting virus or products of the infected cells, such as complement fixing and hemagglutinating antigens. Also, in the steady state of continuous culture, a steady stream of virus is obtained. Several culture methods, adapted to the specific cell/virus systems have been described by *Van Hemert, Kilburn* and *Van Wezel* (1969).

The development of production processes, in homogeneous culture, for virus vaccines, and other virus products, is being studied in our laboratory, using primary cells as well as euploid cell-lines as virus substrates (*Van Wezel,* 1967a; *Van Hemert, Kilburn* and *Van Wezel,* 1969; *Kilburn* and *Van Wezel,* 1970; *Kilburn,* 1969).

Although the technical arrangement is similar to the one described in this thesis, this work constitutes a separate aspect of the unit process of vaccine production. Hence, it will be published in detail in the near future (*Van Wezel*).

CHAPTER 5

General Discussion

This general discussion is an addition to the detailed discussions, given in Chapter 4, on the individual vaccines.

The main difference between this treatise on vaccine production and others, is the way in which data of general applicability are given prior to the consideration of the preparation of any given vaccine. Such a method of treatment eliminates the unnecessary preamble from each separate discussion. In addition it allows full attention to be given to the "basic tools", which feature mainly the apparatus and the general methodology of cultivation. For these reasons we have defined this way of treatment as "Unit Process", although, it is realized that this term is used in a slightly modified significance, compared with the original one of *Lewis.*

One of the basic assumptions underlying this work is that homogeneous cultivation is, both in theory and practice, always to be preferred above non-homogeneous cultivation techniques. It is emphasized that homogeneous culture is defined here as a culture system in which all constituents are uniformly distributed: ideally, this means a complete dispersion of single bacterial or tissue cells in a true solution of all components, including dissolved gases, without detectable concentration gradients. It is recognized that certain deviations from this ideal behaviour must occur in practice. These deviations are small compared with the situation in basically non-homogeneous systems such as a bacterial surface culture. Some of the deviations are fundamental and hence impossible to avoid. The **B.C.G.** strain of **M.tuberculosis,** for instance, is not suspendable as single cells, but disperses only in clumps, on average consisting of at least ten cells. To give another example, the "microcarrier" method used by *Van Wezel,* as a means

to circumvent the inability of primary as well as euploid tissue cells to grow in free suspension gives rise to particles of 100-250 μ in diameter. The advantage of the latter over a massive clump of bacterial cells is that the tissue cells are all situated, like a monolayer, on the outside of the particles.

Addition of components to a culture, such as air in aerated cultures, medium in continuous cultures, and acid or alkali in pH-controlled cultures gives rise to concentration gradients, which may only be temporary. Good mixing reduces the likelihood of a detectable lack of homogeneity, which would thus prevent the attribution of the physico-chemical data, measured by a probe in the culture, to the culture as a whole. However, in **B.C.G.** and tissue cell cultivation we see a further conflicting complication. To avoid sedimentation of the clumps, vigorous stirring is required, but the cells are easily damaged mechanically and thus a compromise has to be taken.

The arguments put forward by those who still advocate basically non-homogeneous systems, can be summarized as their suggested inability to find the conditions in homogeneous culture, which are identical to those existing in that part of a non-homogeneous system which gives rise to formation of the desired product. In our experiments, we have never found such a situation to exist, and are for good reasons unshakably in favour of basically homogeneous culture systems.

The measurement and control of not only temperature, but also pH and pO_2 have become a standard feature of the culture equipment, which we developed. This does not imply that control of pH and pO_2 is invariably necessary, or advantageous. In the cultivation of **B.pertussis**, for instance, pH-control gives neither better growth nor higher yield. However, in this case the pH-rise is a clear indication of the stage of growth, and so pH-recording serves a better purpose than pH-control would do. To give an other example, it is not expected that with the slow growth rate of **B.C.G.**, pO_2-control would improve cultivation. But, here again, the pO_2-recording trace provides a valuable criterion for the determination of the moment of harvest of the culture, which is explained as follows. For the live **B.C.G.** vaccine it is of paramount importance that a high percentage of viable cells is present in the preparation. Because colony-counting is often misleading due to clumping, determination of the oxygen consumption, measured in the *Warburg* apparatus has been advocated. We have now shown that by following the course of pO_2 of the culture, and harvesting at the point of minimal pO_2 a cell suspension of consistently high viability is obtained.

These two examples also have a significance in an other respect. In the literature, the endpoint of cultivation, for a certain purpose, is frequently defined by a certain culture duration (a "x-hour's culture"). Now the condition of the culture after x hours may be dependent on many factors,

including the condition and the amount of inoculum. Therefore duration is by no means a dependable criterion. We have found that for each system, a useful criterion should be sought, which must be experimentally proved to be related to the best time for harvesting.

We found that the measurement of the oxygen tension (pO_2) in the culture was an almost unexplored field, among others because sterilizable oxygen probes were not available. With the probe which we developed it was possible to follow the pO_2-level with even prolonged cultivations such as in continuous cultures. The most remarkable finding from use of this oxygen probe is that good yields are obtained in aerobic processes over a wide range of pO_2 conditions. This is the case for toxin production by **C.diphtheriae**, where toxin yield is independent of the pO_2-level between 0.2 and at least 50 mm Hg (0.1 to 30 % air saturation); oxygen control at 25 mm Hg gives maximal yields in batch as well as continuous culture, whereas, in the absence of oxygen control, almost complete anaerobiosis is rapidly reached, with consequent low toxin yields.

In the cultivation of **V.cholerae** and **S.typhi** oxygen control at 10 % air saturation gave a dramatic yield increase of 200 %. In non pO_2-controlled cultures, the pO_2 soon reached zero under the normal aeration conditions for our cultures. It must be noted that our oxygen control technique, namely by changing the aeration mixture (mostly by admixing pure oxygen) is only feasible in the small volumes used. It would only be economical up to a culture volume of 300-500 litre. An alternative way of oxygen control, using the air flow rate or the stirrer speed as a variable, has also been envisaged, by using a variation of the control apparatus developed. It has not yet been practised by us.

A very interesting phenomenon is reported to occur in the cultivation of **B.pertussis**, although the preliminary findings need further confirmation. It is postulated that the delicate balance between amino-acids (and possibly peptides) is upset by high pO_2-values, oxidizing these compounds and leaving insufficient, or giving a poor mixture, for formation of the protective antigen, which consists mainly of protein. Experiments, in which pO_2 is controlled, from the start at values near 1 mm Hg, are being designed to confirm the hypothesis.

It is obvious that the principle of "steady state" continuous culture comes to the mind of those studying modern cultivation methods. Extensive experiments have been performed with continuous culture of **B.pertussis** and of **C.diphtheriae.** Experimentally, they may be considered as succesful. They have not, however, led to the introduction of this culture method for routine vaccine production. The main reason is that, for the relatively small culture

volumes needed, it is not worth the extra complication of the procedure. Pertussis vaccine has the added difficulty that potency tests take four weeks; it once occurred that a continuous culture had been producing a valueless **B.pertussis** suspension for a whole month before we could detect it !

For studying the mechanism of product formation, continuous culture is, of course, an ideal method. Using continuous culture, the toxinogenesis of **C.diphtheriae** was studied extensively. In collaboration with *Righelato,* it was possible to prove that the inhibition of toxinogenesis by high Fe-concentrations (in the order of magnitude of 100 μM) is almost instantaneous: imposition of a step change in Fe-concentration in a toxin producing continuous culture led to decrease of the toxin concentration almost exactly according to the wash-out curve.

The apparatus developed by us proved, however, highly suitable for the technique of continuous culture. This technique should be reconsidered, where large volumes, in the order of 10.000 litres, would be needed, such as in the preparation of a purified vaccine with a low over-all yield, or for supplying a certain vaccine to a much greater population than ours.

At the time of our decision, a decade ago, to develop in our laboratory a complete system of apparatus for cultivation, storage and mixing in connection with vaccine production, we did not fully anticipate the consequences. Not only has the development gone further, and embraced more aspects of instrumentation than had been foreseen. It has also attracted attention in other institutes. So it is known that diphtheria toxin is produced in the "Bilthoven Unit" in several places (pers.comm. of *Donikian,* Lyon; *Saletti,* Sienna; *Sinković,* Zagreb; *Sugijati Suharto,* Bandung); also, pertussis vaccine is prepared in this apparatus (*Mynard,* 1970; pers.comm. of *Labert,* Paris; *Sunotoredjo,* Bandung). The same applies for cholera and typhoid vaccines. Furthermore, there are several other processes, using the apparatus, among them preparation of veterinary vaccines, performed in several parts of the world, about which no particulars have been communicated to the author. A slight modification of the "Bilthoven Unit" is used to prepare **B.abortus** vaccine (*E.Houwink,* Weesp, pers.comm.).

From the technical point of view, the two main features of the apparatus are the rigid standardization, maintained from the beginning, enabling the interchange of many parts, and the bringing together of all auxiliary apparatus in a panel. This last feature not only gives a certain elegance to use, but, of more fundamental importance, it prevents aseptic work being obstructed by disorganized apparatus.

In summarizing the practical results of our study, we may state that the production of bacterial vaccines and toxins can now take place in the same

basic cultivation apparatus. According to the character of the process, the recording of pH and pO_2, with or without control, is used to obtain an optimal process. The products studied include pertussis-, cholera-, typhoid- and B.C.G.-vaccines and diphtheria-, tetanus- and staphylococcal-α-toxoids. The production of viral vaccines, involving the tissue culture- and the virus multiplication step, was recently shown to be able to follow the same concept of Unit Process.

Summary

The leading thought of this thesis is a consequence of the observation that processes used in the preparation of several vaccines have more features in common than is generally recognized. With reference to the scheme developed by *Lewis* for chemical technology, the approach to vaccine production is called Unit Process.

The application of the concept of Unit Process involved the development of suitable cultivation equipment, which is described in detail. One of the main features, required of this equipment is, that it promotes the optimization of all production processes concerned. Arguments are put forward to prove that homogeneous cultivation in adequately stirred vessels is always preferable over other methods. One of the main reasons is that only in homogeneous systems can measurement of physico-chemical variables be successfully applied. A critical discussion of homogeneity of cultures is also made.

Among the physico-chemical variables considered, the dissolved oxygen tension (pO_2) takes a prominent place. The development of a steam sterilizable probe is described, and also the application of measurement and control of this variable to several processes.

A detailed description is given of research, and development to a practicable production process for several bacterial vaccines. However, only in the production of toxins could a quick and dependable estimation of the amounts produced be made during the cultivation. For the other vaccines a test is either absent, or takes weeks. In the absence of a reliable test, the cell production is considered as a criterion of optimization. Special attention is paid to the determination of the moment of harvest of the culture, to replace the equivocal use of a predetermined cultivation time. The measurement of variables such as pH and pO_2 provided, especially in the absence of a quick test on product formation, a better evaluation of the stage of the process.

Pertussis vaccine is now produced with yields of 60-80 x 10^9 cells/ml, and a mouse protective activity of at least 4 IU per human dose. The endpoint of the culture is related to the pH reached during growth. Extensive research has been done on a special method of harvesting, namely by acid precipitation. The method was abandoned because it produced a more toxic vaccine; the nature of the toxin involved was studied. Dependence of antigen yield on the level of dissolved oxygen (pO_2) during the culture is postulated but needs further investigation. Continuous culture has been applied, but

was abandoned eventually for two reasons: occasional phenotypic variation leading to a culture producing less antigen, and the presence of dark particles (lysed cells) in the effluent.

In the production of cholera and typhoid vaccine, cell yield was the main criterion. The use of pO_2-control caused the yield to increase from 30 to 100×10^9 cells/ml for both vaccines. The glucose addition to the growing culture was adapted in a special way in order to avoid formation of rough cells. pH-control proved useful. A detailed description is given of a relatively new method of processing typhoid vaccine, i.e. by drying of a suspension in acetone.

B.C.G.-bacteria have never been cultivated before in stirred vessels. One of the specific difficulties encountered was the clumping of the cells in suspension, thus preventing cell counting by the plating method, which is considered necessary by many producers of this live vaccine. Good yields were obtained in homogeneous culture in media with a surfactant like Tween-80 or Triton. Cell counting was replaced by *Warburg* measurements in the live suspension. The time of harvest of the culture was related to the minimum in pO_2-level.

Toxin formation by **C.diphtheriae** was intensively studied and eventually led to a production process with a toxin yield of 200-400 Lf/ml. Important parameters for this production are the pO_2-level during cultivation, and the iron concentration of the culture. Also, the papain digestion of the meat, as an integrating step in the preparation of the culture medium, proved to be important. Continuous cultivation was developed for the following two purposes. The suitability of this method for production was proved, and complete success was achieved. Constant yields of 200 Lf/ml were obtained over long periods of time. The second purpose was the kinetic study of toxinogenesis, in its relation to variables such as iron concentration and pO_2. Interesting results were obtained.

For tetanus toxin production, a special cultivation vessel was developed, used not only for cultivation but also for medium preparation, and for pressing the grown culture through a Seitz-filter. Maximal yields of 60-80 Lf/ml were only obtained when air was blown over the culture. The factors influencing the gradual diffusion of the toxin from the cell were studied. An incinerator was especially developed for sterilization and deodorization of the spent gas of this culture.

The most interesting development in the study of the α-toxin formation by **St.aureus** was the determination of the optimal gas mixture for production.

The application of the Unit Process principle to the production of viral vaccines, involving the tissue cell cultivation and the virus multiplication, is briefly discussed, but no experimental work is mentioned.

Samenvatting

Het kernthema van dit proefschrift is geïnspireerd op de constatering dat de processen, toegepast voor de bereiding van verschillende vaccins, vaak veel nauwer met elkaar verwant zijn dan men op het eerste gezicht aanneemt. Voor de door ons gevolgde benadering is, in navolging van de door *Lewis* voor de chemische technologie gebruikte terminologie, de benaming "Unit Process" gekozen.

De toepassing van het concept van "Unit Process" impliceerde de ontwikkeling van geschikte kweekapparatuur; deze wordt uitvoerig beschreven. Een van de belangrijkste aan deze apparatuur te stellen eisen was, dat het de optimalisering van alle betrokken bereidingsprocessen mogelijk maakt. Bewijzen worden aangevoerd voor de stelling dat de homogene kweekmethode, in goed geroerde kulturen, altijd de voorkeur verdient. Een van de voornaamste argumenten hiervoor is dat fysisch-chemische variabelen alleen in homogene systemen zinvol gemeten kunnen worden. Het begrip homogeniteit wordt aan een kritische discussie onderworpen.

De zuurstofspanning in de oplossing (pO_2) neemt onder de toegepaste fysisch-chemische variabelen een speciale plaats in. De ontwikkeling van een met stoom steriliseerbaar meetelement wordt beschreven, evenals de toepassing van meten en regelen van deze variabele op verscheidene processen.

Van verscheidene vaccins wordt zowel de research als de ontwikkeling tot een bruikbaar bereidingsproces gedetailleerd beschreven. De bepaling van de geproduceerde hoeveelheden tijdens de kweek was alleen mogelijk bij de bereiding van toxines, daar hiervoor snelle en betrouwbare bepalingsmethoden bestaan. Voor andere vaccins is zulk een bepaling òf geheel afwezig, òf neemt weken in beslag. Alleen als er geen bepaling van de werkzame stof mogelijk was, werd de celproduktie als leidraad voor optimalisering gebruikt.

Aan de bepaling van het juiste moment van beëindiging van de cultuur werd bijzondere aandacht besteed. Het gebruik van een vooraf bepaalde kweekduur werd als dubbelzinnig verworpen. Het benutten van variabelen zoals pH en pO_2 verschafte een beter inzicht in het proces, met name in die gevallen, waar geen snelle werkzaamheidstest voorhanden is.

Kinkhoestvaccin wordt thans geproduceerd met een opbrengst van 60-80 x 10^9 cellen/ml, en een werkzaamheid, gemeten in de muisbeschermingstest, van minstens 4 IU per menselijke dosis. Het eindpunt van de cultuur wordt hierbij in verband gebracht met de tijdens de groei bereikte

pH. Veel speurwerk werd verricht inzake een bijzondere wijze van oogsten, namelijk door zuurprecipitatie. De methode werd verworpen toen bleek dat het geproduceerde vaccin toxischer was; de aard van het toxine in kwestie werd bestudeerd. Er werd een theorie ontwikkeld over de samenhang tussen pO_2 en antigeenopbrengst, maar verder onderzoek hierover is geboden. Continue cultuur werd geprobeerd, maar uiteindelijk toch niet toegepast om twee redenen: ten eerste bleken af en toe phenotypische veranderingen op te treden, die leidden tot een kweek met een lagere antigeenproduktie; ten tweede kwamen donkere stukjes (gelyseerde cellen) in de afgetapte vloeistof voor, die moeilijk te verwijderen waren.

Bij de bereiding van cholera- en tyfusvaccin was celopbrengst het voornaamste criterium. Toepassing van pO_2-regeling gaf een verhoging van de opbrengst van 30 tot 100 x 10^9 cellen/ml bij beide vaccins. De toevoeging van glucose aan de kweek moest op speciale wijze aan de omstandigheden worden aangepast, teneinde de vorming van rough-cellen tegen te gaan. pH-regeling bleek van voordeel te zijn. Een nieuwe methode om tyfusvaccin op te werken, namelijk door drogen van een acetonsuspensie, wordt uitvoerig beschreven.

Voor zover bekend, zijn **B.C.G.**-bacteriën nooit tevoren in geroerde vaten gekweekt. Een specifieke moeilijkheid was de eigenschap om in suspensie cel-aggregaten te vormen; hierdoor was het weinig zinvol om tellingen van levende cellen op platen te verrichten, hetgeen door vele onderzoekers als noodzakelijk wordt beschouwd voor de evaluatie van dit levende vaccin. Goede opbrengsten werden behaald in homogene kweek, wanneer aan de voedingsbodem een oppervlakte-actieve stof zoals Tween-80 of Triton werd toegevoegd. Het tellen van kolonies werd vervangen door *Warburg*-bepalingen in de levende suspensie. Het eindpunt van de kweek werd in verband gebracht met het minimum in de pO_2-curve.

Een uitgebreide studie werd gemaakt van de toxinevorming door **C.diphtheriae.** Met de verkregen gegevens werd een produktiemethode opgesteld die een toxine-opbrengst van 200-400 Lf/ml waarborgt. Belangrijke parameters van het kweekproces zijn het pO_2-niveau en de ijzerconcentratie in de kweek. De vertering door middel van papaine, die een essentiële stap in de bereiding van de voedingsbodem vormt, vereiste bestudering. Ook werd een continue kweekmethode ontwikkeld, om tweeërlei redenen: ten eerste om de geschiktheid van deze methode voor produktie te onderzoeken. In dit opzicht werd volledig succes bereikt: een constante opbrengst van 200 Lf/ml gedurende een lange periode bleek mogelijk. De tweede reden was de studie van de kinetica van toxinevorming in samenhang met variabelen als ijzerconcentratie en pO_2. Ook in dit opzicht werden interessante resultaten bereikt.

Een speciaal kweekvat werd ontwikkeld voor de bereiding van tetanus-

toxine. Hierin wordt niet alleen gekweekt, maar ook de voedingsbodem bereid; tenslotte wordt de ketel ook als drukvat voor de Seitz-filtratie benut. De beste resultaten werden bereikt wanneer lucht over de cultuur werd geblazen. De factoren, die de geleidelijke diffusie van het toxine uit de cel beheersen werden bestudeerd. Tenslotte werd een verbrandingskamer ontworpen, met het speciale doel het uit de cultuur tredende gas kiem- en reukvrij te maken.

Het interessantste punt bij de bestudering van de α-toxinevorming door **St.aureus,** bleek het gasmengsel te zijn, dat vereist is voor optimale produktie.

Als laatste onderwerp is de mogelijke toepassing van het Unit Process principe op de bereiding van virusvaccins behandeld. Hierbij gaat het dus om de kweek van weefselcellen en de vermeerdering van virussen. In de korte hieraan gewijde beschouwing zijn geen proeven genomen.

References

Alouf,J.E., 1958, Ph.D.Thesis, Univ.Paris,Fac.of Pharmacy, Series E.nr.60

Bänffer,J.R.J., 1961, Thesis, Utrecht

Barker,H.A., 1944, Proc.nat.Acad.Sci.(Wash.), **30,** 88

Barker,H.A. and *Kamen,M.D.,* 1945, Proc.nat.Acad.Sci.(Wash.), **31,** 219

Bartholomew,W.H.; Karow,E.O. and *Sfat,M.R.,* 1950, Ind.Eng.Chem., **42,** 1827

Behring,E.von, 1913, Dtsch.med.Wschr., **39,** 873

Behring,E.von and *Kitasato,S.,* 1890, Dtsch.med.Wschr., **16,** 1113

Behring,E.von and *Wernicke,* 1892, Z.Hyg.Infekt.-Kr., **12,** 10

Benenson,A.S. et *al.,* 1964, Bull.Wld Hlth Org., **30,** 635

Benenson,A.S. and *Spaun,J.,* 1964, Bull.Wld Hlth Org., **30,** 647

Billaudelle,H.; Hedén,C.G. and *Malmgren,B.,* 1959, J.biochem.microbiol.Technol.Engng., **1,** 173

Blachère,H. and *Jamart,G.,* 1969, Biotechnol.Bioeng., **11,** 1005

Bordet,J. and *Gengou,O.,* 1906, Ann.Inst.Past., **20,** 731

Borkowski,J. and *Johnson,M.J.,* 1967, Biotechnol.Bioeng., **9,** 635

Box,G.E.P., 1957, Appl.Statist., **6,** 81

Brandwijk,A.C., 1926, Thesis, Utrecht

Brandwijk,A.C.; Cohen,H.H.; Hekker,A.C.; Hofman,B.; Ramshorst,J.D.van; Tasman,A.; Driel,C.B.van; Schaapman-Tilma,B. and *Vries,J.A.van,* 1961, Ned.T.Geneesk., **105,** 1331

Brown,H.C., 1913–14, Indian J.med.Res., **1,** 711

Brown,W.E. and *Peterson,W.H.,* 1950 a, Ind.Eng.Chem., **42,** 1769

Brown,W.E. and *Peterson,W.H.,* 1950 b, Ind.Eng.Chem., **42,** 1823

Burnet,F.M., 1930, J.Path.Bact., **33,** 1

Callow,D.S. and *Pirt,S.J.,* 1961, J.appl.Bact., **24,** 12

Capstick,P.B.; Telling,R.C.; Chapman,W.G. and *Stewart,D.L.,* 1962, Nature, **195,** 1163

Carle and *Rattone,* 1884, G.Accad.Med.Torino 3d Ser., **32,** 174

Casman,E.P., 1940, J.Bact., **40,** 601

Chain,E.G.; Paladino,S.; Callow,D.S.; Ugolini,F. and *Sluis,J.van der,* 1952, Bull.Wld Hlth Org., **6,** 73

Chain,E.B.; Paladino,S.; Ugolini,F.; Callow,D.S. and *Sluis,J.van der,* 1954, R.C.Inst.sup.Sanità, **17,** 61

Cockburn,W.C., 1955, Bull.Wld Hlth Org., **13,** 395

Cohen,H.H., 1968, Arch.roum.path.exp., **27,** 33

Cohen,H.H. and *Hemert,P.A.van,* 1962, Round Table Conference Perm.Sect.immunobiol. Standard.Prague, 24

Cohen,S.M. and *Wheeler,M.W.,* 1946, Amer.J.publ.Hlth, **36,** *371*

Cooper,C.M.; Fernstrom,G.A. and *Miller,S.A.,* 1944, Ind.Eng.Chem., **36,** 504

Cvjetanović,B. and *Uemura,K.,* 1965, Bull.Wld Hlth Org., **32,** 29

Descombey,P., 1924, C.R.Soc.Biol.(Paris), **91,** 239

Dispigno,J.E., 1961, Sci.Rep.Ist.sup.Sanità, **1,** 232

Dubos,R.J. and *Fenner,F.,* 1950, J.exp.Med., **91,** 267

Dubos,R.J. and *Middlebrook,G.*, 1947, Amer.Rev.Tuberc., **56**, 324
Earle,W.R. et *al.*, 1954, Ann.N.Y.Acad.Sci., **58**, 1000
Eberth,C.J., 1880, Virchows Arch.path.Anat., **81**, 58
Edsall,G., 1961, Publ.Hlth Rep.(Wash.), **76**, 811
Edwards,D.C., 1960, J.gen.Microbiol., **22**, 698
Edwards,D.C. and *Seamer,P.A.*, 1960, J.gen.Microbiol., **22**, 705
Elsworth,R.; Capell,G.H. and *Telling,R.C.*, 1958, J.appl.Bact., **21**, 80
Elsworth,R.; Meakin,L.R.P.; Pirt,S.J. and *Capell,G.H.*, 1956, J.appl.Bact., **19**, 264
Elsworth,R.; Morris,E.J. and *East,D.N.*, 1961, Chem.Engin., **157**, A47
Falini,R., 1960, The Pilot-plant for Chemical Microbiol. of the Instituto Superiore di Sanità, Roma
Felix,A., 1941, Brit.med.J., 1941i, 391
Felix,A. and *Pitt,R.M.*, 1934, J.Path.Bact., **38**, 409
Feustel,I.C. and *Humfeld,H.*, 1964, J.Bact., **52**, 229
Fiechter,A., 1962, Chem.Ing.Tech., **10**, 692
Fiechter,A. and *Meyenburg,K.von*, 1968, Biotechnol.Bioeng., **10**, 535
Flynn,D.S.; Kilburn,D.G.; Lilly,M.D. and *Webb,F.C.*, 1967, Biotechnol.Bioeng, **9**, 623
Fortune,W.B.; MacConnick,S.L.; Rhodehamel,Jr.H.W. and *Stefaniak,I.J.*, 1950, Ind.Eng.Chem., **42**, 191
Francis,Jr.T.; Korns,R.F.; Voight,R.B.; Brisen,M.; Hemphill,F.M.; Napier,J.A. and *Tolchinsky,E.*, 1955, Amer.J.publ.Hlth, **45**, Suppl.
Friedland,W.C.; Peterson,M.H. and *Sylvester,J.C.*, 1956, Ind.Eng.Chem., **48**, 2180
Fuld,G.J. and *Dunn,C.G.*, 1958, Appl.Microbiol., **6**, 15
Gaffky, 1884, Mitt.Kaiserl.Gesundheitsamte, **2**, 372
Geser,G. 1970, Symp.Ser.immunobiol.Stand., **13**, 36
Gladstone,G.P., 1938, Brit.J.exp.Path., **19**, 208
Gladstone,G.P. and *Glencross,E.J.G.*, 1960, Brit.J.exp.Path., **41**, 313
Gleichmann,U. and *Lübbers,D.W.*, 1960, Pflügers Arch.ges.Physiol., **271**, 341
Glenny,A.T. and *Allen,K.*, 1921, J.Path.Bact., **24**, 61
Glenny,A.T. and *Hopkins,B.E.*, 1913, Brit.J.exp.Path., **4**, 283
Goldner,M.; Jakus,C.M.; Rhodes,H.K. and *Wilson,R.J.*, 1966, J.gen.Microbiol., **44**, 439
Gori,G.B., 1965, Appl.Microbiol., **13**, 909
Gray,D.G., 1946, Aust.J.exp.Biol.med.Sci., **24**, 301
Haffkine, 1892 a, C.R.Soc.Biol.(Paris), **4**, 635
Haffkine, 1892 b, C.R.Soc.Biol.(Paris), **4**, 671
Hansford,G.D. and *Humphrey,A.E.*, 1966, Biotechnol.Bioeng., **8**, 85
Harrison,D.E.F., 1971, 5th international Symposium on continuous Culture of Micro-organisms, Oxford, (in press)
Hayflick,L., 1967, 1st Scient.Public. Pan Americ.Health Org. nr. 147
Hayflick,L. and *Moorhead,P.S.*, 1961, Exp.Cell Res., **25**, 585
Hedén,C.G., 1957, Nature, **179**, 324
Hedén,C.G., 1958, Nord.Med., **31**, 1090
Hedén,C.G., 1962, Abstract C22S, Internat.Congr.Microbiol., Montreal
Hedén,C.G. and *Holme,T.*, 1961, Continuous Culture of Microorganisms Symposium, London, 1960, Soc.Chem.Ind.Monograph., **12**, 118
Hedén,C.G. and *Malmborg,H.S.*, 1958, Acta path.microbiol.scand., **44**, 405
Hejfec,L.B.; Levina,L.A.; Kuzminova,M.L.; Salmine,L.V.; Slavina,A.M. and *Vasileva,A.V.*, 1968, Bull.Wld Hlth Org., **38**, *907*
Hemert,P.A.van, 1958, Abstr.VII Internat.Congr.Microbiol., *Stockholm, 22m*

Hemert,P.A.van, 1962 a, Abstr.VIII Internat.Congr.Microbiol., Montreal
Hemert,P.A.van, 1962 b, Ant.van Leeuwenhoek, **28,** 239
Hemert,P.A.van, 1962 c, Versl.Meded.Volksgezondh., 329
Hemert,P.A.van, 1964 a, Versl.Meded.Volksgezondh., 271
Hemert,P.A.van, 1964 b, Biotechnol.Bioeng., **6,** 381
Hemert,P.A.van, 1964 c, Global.Imp.Appl.Microbiol., Stockholm, 507
Hemert,P.A.van, 1964 d, 2nd International Fermentation Symposium, London
Hemert,P.A.van, 1967, Versl.Meded.Volksgezondh., 169
Hemert,P.A.van, 1969, Progr.immunobiol.Standard., **3,** 297
Hemert,P.A.van, 1970, Symp.Ser.immunobiol.Standard., **13,** 39
Hemert,P.A.van; Kilburn,D.G.; Righelato,R.C. and *Wezel,A.L.van,* 1969, Biotechnol. Boeing., **11,** 549
Hemert,P.A.van; Kilburn,D.G. and *Wezel,A.L.van,* 1969, Biotechnol. Bioeng., **11,** 875
Hemert,P.A.van and *Wezel,A.L.van,* 1966, Symp.Ser.immunobiol.Standard., **3,** 221
Hemert,P.A.van; Wezel,A.L.van and *Cohen,H.H.,* 1964, Nature, **203,** 774
Herbert,D., 1959 Recent Progress in Microbiology, (VII Internat.Congr.Microbiol., Stockholm, 1958), 381
Herbert,D., 1961, Sci.Rep.Ist.sup.Sanità, **1,** 265
Holt,L.B., 1947, Lancet, **252,** 282
Hornibrook,J.W., 1939, Publ.Hlth Rep.(Wash.), **54,** 1847
Hornibrook,J.W., 1940, Proc.Soc.exp.Biol.(N.Y.), **45,** 598
Humfeld,H.J., 1947, J.Bact., **54,** 689
Jacob,H.E., 1970 a, Path.Microbiol., **36,** 57
Jacob,H.E., 1970 b, Meth. in Microbiol., **2,** 91, ed. by Norris,J.R. and Ribbons,D.W., Acad.Press
Jebb,W.H.H. and *Tomlinson,A.H.,* 1955, J.gen.Microbiol., **13,** 1
Johnson,M.J.; Borkowski,J. and *Engblom,C.,* 1964, Biotechnol.Bioeng., **6,** 457
Joó,I.; Pusztai,S.; Maté,J.; Réthy,L.; Eckhardt,E. and *Juhász,V.,* 1964, Z.Immun.-Allergie-Forsch., **127,** 230
Joó,I.; Zalay,L.; Kiss,I.; Füvessy,I. and *Pusztai,S.,* 1959, Zbl.Bakt.I.Abt.Orig., **176,** 496
Kendrick,P.L.; Eldering,G.; Dixon,M. and *Misner,J.,* 1947, Amer.J.publ.Hlth, **37,** 803
Kilburn,D.G., 1969, Versl.Meded.Volksgezondh., 287
Kilburn,D.G. and *Wezel,A.L.van,* 1970, J.gen.Virol., **9,** 1
Kitasato,S., 1889, Z.Hyg.Infekt.-Kr., **7,** 225
Koch,R., 1882, Berl.klin.Wschr., **19,** 221
Koch,R., 1886, New Sydenham Soc., **115,** 327
Koch,R., 1897, Dtsch.med.Wschr., **23,** 209
Kolle,W., 1896, Zbl.Bakt.I.Abt.Orig., **19,** 97
Kroll,C.L.; Formanek,S.; Covert,A.S.; Cutter,L.A.; West,J.M. and *Brown,W.E.,* 1956, Ind.Eng.Chem., **48,** 2190
Kurokawa,M.; Iwasa,S. and *Ishida,S.,* 1965, Jap.J.med.Sci.Biol., **18,** 161
Lane,A.G., 1970, Appl.Microbiol., **19,** 512
Latham,W.C.; Bent,D.F. and *Levine,L.,* 1962, Appl.Microbiol., **10,** 146
Leonard,G.F. and *Holm,A.,* 1935, J.Immunol., **29,** 209
Leslie,P.H. and *Gardner,A.D.,* 1931, J.Hyg., **31,** 423
Lewis,W.K., 1923, Principles of Chemical Engineering
Lewis,W.K., 1923, in *Walker,W.H.; Lewis,W.K. and Adams,W.H.,* Principles in Chemical Engineering, Mac Graw-Hill, New York
Linggood,F.V. and *Fenton,E.L.,* 1947, Brit.J.exp.Path.; **28,** 354

Loeffler,F., 1884, Mitt.Kaiserl. Gesundheitsamte, **2**, 421
Lumb,M. and *Fawcett,R.*, 1951, J.appl.Chem., **1**, S,94
Maaløe,O., 1955, Bull.Wld Hlth Org., **12**, 769
MacFarlane,J., 1907, J.Amer.med.Ass., **49**, 1176
Mackereth,F.J.H., 1964, J.Sci.Instr., **41**, 38
MacLennan,D.G. and *Pirt,S.J.*, 1965, Chem.Ind., 1973
MacLennan,D.G. and *Pirt,S.J.*, 1966, J.gen.Microbiol., **45**, 289
MacPherson,I. and *Stoker,M.*, 1962, Virology, **16**, 147
Madsen,T., 1933, J.Amer.med.Ass., **101**, 187
Málek,I., 1961, Sci.Rep.Ist.sup.Sanità, **1**, 279
Málek,I. and *Řičica,J.*, 1970 a, Folia microbiol.(Praha), **15**, 129
Málek,I. and *Řičica,J.*, 1970 b, Folio microbiol.(Praha), **15**, 377
Malmgren,B. and *Hedén,C.G.*, 1952 a, Acta path.microbiol.scand., **30**, 223
Malmgren,B. and *Hedén,C.G.*, 1952 b, Acta path.microbiol.scand., **30**, 284
Mancy,K.H.; Okun,D.A. and *Reilley,C.N.*, 1962, J.electroanal.Chem., **4**, 65
Medical Research Council, 1959, Brit.med.J., 1959i, 994
Medical Research Council, 1956, Brit.med.J., 1956ii, 454
Monod,J., 1950, Ann.Inst.Past., *79*, 390
Mueller,J.H. and *Miller,P.A.*, 1945, J.Immunol., **50**, 377
Mueller,J.H. and *Miller,P.A.*, 1947, J.Immunol., **56**, 143
Mueller,J.H. and *Miller,P.A.*, 1954, J.Bact., **67**, 271
Mukerjee,S., 1963, Bull.Wld Hlth Org., **29**, 753
Mynard,M.C., 1970, Symp.Ser.immunobiol.Standard., **13**, 58
Nagel,J., 1970, Thesis, Utrecht, 31
Nelson,H.A.; Maxon,W.D. and *Elferdink,T.H.*, 1956, Ind.Eng.Chem., **48**, 2183
Nicolaier,A., 1884, Dtsch.med.Wschr., **10**, 842
Nicolaier,A., 1886, Jber.Fortschr.path.Mikroorg., **2**, 270
Novick,A. and *Szilard,L.*, 1950, Proc.Nat.Ac.Sci., **36**, 708
Pappenheimer,A.M., 1955, Symp.Soc.gen.Microbiol., **5**, 40
Pappenheimer,A.M. and *Hendee,E.D.*, 1947, J.biol.Chem., **167**, 701
Pappenheimer,A.M.; Miller,P.A. and *Yoneda,M.* 1962, J.gen.Microbiol., **28**, 531
Parish,H.J. and *Clark,W.H.M.*, 1931, J.Path.Bact., **34**, 593
Parker,J.T.; Hopkins,J.G. and *Gunther,A.*, 1925–1926, Proc.Soc.exp.Biol.(N.Y.), **23**, 344
Peetoom,F. and *Veer,M.van der*, 1967, Proc.Internat.Conf.Tetanus, Berne, 1966, 237
Perry,H.M.; Findlay,H.T. and *Bensted,H.J.*, 1933, J.roy.Army med.Cps., **60**, 241
Perry,H.M.; Findlay,H.T. and *Bensted,H.J.*, 1934, J.roy.Army med.Cps., **63**, 1
Proom,H., 1955, J.Gen.Microbiol., **12**, 63
Pusztai,Z.; Joó,I. and *Juhász,V.P.*, 1961, Z.Immun.-Forsch., **122**, 278
Ramon,G., 1923, C.R.Soc.Biol.(Paris), **89**, 2
Ramon,G., 1928, Ann.Inst.Past., **42**, 959
Ramshorst,J.D.van, 1951, Thesis, Amsterdam
Ramshorst,J.D.van, 1963, Ant.van Leeuwenhoek, **29**, 101
Ramshorst,J.D.van, 1969, Progr.immunobiol.Standard., **3**, 324
Raynaud,M., 1951, Ann.Inst.Past., **80**, 356
Raynaud,M.; Alouf,J. and *Mangalo,R.*, 1959, Ann.Inst.Past., **96**, 276
Raynaud,M.; Turpin,A.; Mangalo,R. and *Bizzini,B.*, 1954, Ann.Inst.Past., **87**, 599
Raynaud,M.; Turpin,A.; Mangalo,R. and *Bizzini,B.*, 1955, Ann.Inst.Past., **88**, 24
Relyveld,E.H.; Raynaud,M.; Bepoldin,O. and *Vais,A.*, 1964, Ann.Inst.Past., **107**, 618
Řičica,J., 1958, Continuous Culture of Microorganisms Symposium, Prague, 75

Righelato,R.C., 1969 a, Versl.Meded.Volksgezondh., 274
Righelato,R.C., 1969 b, J.gen.Microbiol., **58**, 411
Righelato,R.C. and *Hemert,P.A.van*, 1968, Proc.4th Continuous Culture of Microorganisms Symposium, Prague, 437
Righelato,R.C. and *Hemert,P.A.van*, 1969, J.gen.Microbiol., **58**, 403
Ring,K.; Schlecht,S., Eschweiler,W. and *Kutscher,J.*, 1969, Arch.Mikrobiol., **65**, 48
Rivett,R.W.; Johnson,M.J. and *Peterson,W.H.*, 1950, Ind.Eng.Chem., **42**, 188
Rogers,H.J., 1954, J.gen.Microbiol., **10**, 209
Rogers,H.J., 1956, Ann.N.Y.Acad.Sci., **65**, 132
Roux,E. and *Yersin,A.*, 1888, Ann.Inst.Past., **2**, 629
Rowatt,E., 1957 a, J.gen.Microbiol., **17**, 279
Rowatt,E., 1957 b, J.gen.Microbiol., **17**, 297
Sanyal,R.K., 1960, Nature, **185**, 537
Seibert,F.B.; Aronson,J.D.; Reichel,J.; Clark,L.T. and *Long,E.R.*, 1934, Amer.Rev. Tuberc., **38**, 399, Suppl.
Sirks,J.L., 1956, Versl.Meded.Volksgezondh., 947
Slezák,J. and *Sikyta,B.*, 1961, J.biochem.microbiol.Technol.Engng., **3**, 357
Solomons,G.L.; 1967, Process Biochem., March
Solomons,G.L.; 1971, Process Biochem., Aug. 36
Spaun,J., 1962 a, Bull. Wld Hlth Org., **26**, 213
Spaun,J., 1962 b, Bull. Wld Hlth Org., **26**, 219
Stainer,D.W., 1970, Symp.Ser.immunobiol.Standard., **13**, 89
Sybesma,Th. and *Bleiker,M.A.*, 1971, B.C.G. Vaccine Symposium, Frankfurt/Main, 1970, Progr.immunobiol.Standard., (in press)
Sykes,J., 1966, Symposium microbiol.Physiol.cont.Cult., Porton
Tasman,A., 1959 a, Ann.Inst.Past., **97**, 835
Tasman,A., 1959 b, Rapport A 1181 Nederlandse Stichting voor Statistiek, 's Gravenhage
Tasman,A., 1960, Versl.Meded.Volksgezondh., 1963
Tasman,A., 1964, Versl.Meded.Volksgezondh., 1964
Tasman,A. and *Brandwijk,A.C.*, 1937, Ant.van Leeuwenhoek, **4**, 187
Tasman,A. and *Brandwijk,A.C.*, 1938, J.infect.Dis., **63**, 10
Tasman,A. and *Brandwijk,A.C.*, 1940, J.Infect.Dis., **67**, 282
Tasman,A. and *Slot,J.van der*, 1953, Ant.van Leeuwenhoek, **19**, 256
Telling,R.C. and *Elsworth,R.*, 1965, Biotechnol.Bioeng., **7**, 417
Telling,R.C.; Radlett,P.J. and *Mowat,G.N.*, 1967, Biotechnol.Bioeng., **9**, 257
Telling,R.C. and *Stone,C.J.*, 1964, Biotechnol.Bioeng., **6**, 147
Turgueniev,I., 1857, Letter to Leo Tolstoi
Typhoid Panel, U.K.Dept. of Techn.Cooper., 1964, Bull. Wld Hlth Org., **30**, 631
Ungar,J.; Muggleton,P.W. and *Dudley,J.A.R.*, 1962, Brit.med.J., 1962**ii**, 1086
Ungar,J.; Stanić,M.; Varallyay,S. and *Chariatte,N.*, 1971, Symp.Ser.immunobiol. Standard., **15**, 197
Updike,S.J. and *Hicks,G.P.*, 1967, Nature, **214**, 986
Vaheri,A.; Sedwick,W.D.; Plotkin,S.A. and *Maes,R.*, 1965, Virology, **27**, 239
Verwey,W.F. and *Sage,D.M.*, 1945, J.Bact., **49**, 520
Verwey,W.F.; Thiele,E.H.; Sage,D.M. and *Schuchardt,L.F.*, 1949, J.Bact., **58**, 127
Wezel,A.L.van, 1967 a, Nature, **216**, 64
Wezel,A.L.van, 1967 b, Versl.Meded.Volksgezondh., 2966
Wezel,A.L.van and *Hemert,P.A.van*, 1965, Versl.Meded.Volksgezondh., 1920
Wezel,A.L.van and *Hemert,P.A.van*, 1967 a, Versl.Meded.Volksgezondh., 151

Wezel,A.L.van and *Hemert,P.A.van,* 1967 b, Versl.Meded.Volksgezondh., 184
Wilson,R.J., 1963, Canad.J.publ.Hlth, **54,** 518
Woiwod,A.J., 1954, J.gen.Microbiol., **10,** 509
Woodin,A.M., 1959, Biochem.J., **73,** 225
Woodin,A.M., 1960, Biochem.J., **75,** 158
World Health Organization, Techn.Rep.Ser., 1964, **293,** 42
World Health Organization, 1968, Current Data: whooping cough, Wld Hlth Statist.Rep., **21,** 124
Wright,A.E. and *Semple,D.,* 1897, Brit.med.J., 1897i, 256
Yugoslav Typhoid Commission, 1964, Bull.Wld Hlth Org., **30,** 623

Acknowledgements

I am obliged to many persons at the Rijks Instituut voor de Volksgezondheid, in addition to those mentioned already by name because of their direct involvement in the work described in the thesis; a.o:

- The laboratory personnel of the Laboratory for Vaccine Production for technical assistance in laboratory experiments.
- Miss J.Koppelman for invaluable help in the preparation of the manuscript.
- Mr.B. van der Laan for drawing many figures.
- The Laboratory for Biological Standardization (head Dr.J.D. van Ramshorst), for carrying out many animal tests.
- The Library for checking the references.
- The Technical Service Department for building part of the apparatus developed.

From the contributions from outside the R.I.V. I want to mention:

- Miss M.Watts Evans for criticizing the English language of the text.
- Ir.H.A.Behagel for his scientific criticism on the manuscript.
- Guus van Hemert for the design of the cover.
- N.V.Apparatenfabriek Van Doorn for building many prototypes.

Curriculum Vitae

Paul van Hemert werd op 29 oktober 1925 te Voorburg geboren, alwaar hij lager onderwijs genoot.

Daarna bezocht hij het Aloysius College te 's-Gravenhage en behaalde daar in 1944 het einddiploma Gymnasium-β.

Na een jaar van gedwongen rust, begon hij in 1945 de studie van scheikundig ingenieur aan de Technische Hogeschool te Delft. Tijdens deze studie was hij gedurende een jaar assistent aan het Laboratorium voor Analytische Scheikunde. Voor het behalen van het ingenieursdiploma in 1952, bewerkte hij bij Prof.Dr.A.J.Kluyver een afstudeeronderwerp op het gebied van de bereiding van antibiotica.

Van 1952 tot 1953 was hij werkzaam op het Laboratorium van de N.V. P.de Gruyter en Zoon te 's-Hertogenbosch. Van 1954 tot 1959 was hij hoofd van het Microbiologisch Laboratorium van de N.V.Organon te Oss, waar hij onder andere een studie maakte van de microbiologische omzetting van steroïden.

Op 1 mei 1959 volgde zijn benoeming bij het Rijks Instituut voor de Volksgezondheid te Bilthoven, met als taak de ontwikkeling van de vaccinbereidingsmethoden. Hiertoe maakte hij een aantal studiereizen onder andere naar de Verenigde Staten, Engeland en Scandinavië. In 1970 bracht hij als medical consultant van de World Health Organization een bezoek aan Bandung, Indonesia, teneinde advies over de produktie van vaccins te geven.

Hij is thans hoofd van het Laboratorium voor Vaccinbereiding aan het Rijks Instituut voor de Volksgezondheid.

STELLINGEN

I

De reproduceerbare bereiding van een bacterieel of viraal produkt is in een homogene cultuur altijd beter gewaarborgd dan in een principieel niet-homogene.

Kluyver,A.J. and *Perquin,L.H.C.*, 1933, Biochem.Z., **266**, 68
Dit proefschrift, p. 17 sqq.

II

De meting van de redox-potentiaal in een bacteriecultuur is in de meeste gevallen van geringe waarde voor de beoordeling van de toestand van de cultuur.

Harrison,D.E.F., 1971, 5th Int.Symp.Cont.Cult.-Microorg., Oxford (in press)
Dit proefschrift, p. 60

III

De kweekduur van een bacteriecultuur is, zelfs bij zo goed mogelijke standaardisatie van de omstandigheden, een onbetrouwbaar kriterium voor de bepaling van het tijdstip van oogsten, indien gestreefd wordt naar optimalisering ten aanzien van kwaliteit en kwantiteit van het beoogde produkt.

Dit proefschrift, p. 70

IV

De waarde van de "viable count" methode, bij de evaluatie van **B.C.G.** vaccin wordt door sommige producenten overschat. De bepaling van het zuurstofverbruik volgens de manometrische methode van *Warburg* is zinvoller.

Sievers,O. and *Sievers,J.*, 1961, Scand.J.clin.Lab.-Invest., **13**, suppl. 60, 9
Lamensans,A. and *Bretey,J.*, 1967, Ann.Inst.Past., **112**, 342
Dit proefschrift, p. 113 sqq.

V

Bij de beoordeling van de gelijkheid van de troebeling van een bacteriesuspensie enerzijds, en een als standaard fungerende suspensie, zoals de W.H.O. International Reference Preparation for Opacity, anderzijds, is een foto-electrisch meetinstrument betrouwbaarder dan het menselijk oog.

Panel Disc. on Vacc. Prep., 1970, Symp.Ser.-immunobiol.Standard., **13**, 55
Dit proefschrift, p. 66

VI

Het zuurstofverbruik van vissen is, per eenheid van gewicht, ongeveer een factor 10.000 lager dan dat van bacteriën. Daarom is, voor de realisering van een bepaalde O_2-spanning in water met vissen, zoals vereist in bepaalde toxicologische proeven, een complete pO_2-regeling overbodig. Volstaan kan worden met het doorleiden van gas met de gewenste zuurstofspanning.

Lloyd,R., 1961, J.exp.Biol., **38**, 447
O'Hara,J., 1971, Water Res., **5**, 143
Dit proefschrift, p. 39 and 54 sqq.

VII

Bij de microbiologische bepaling van vitaminen is, op theoretische gronden, een betere correlatie van de vitamine-concentratie met de groeisnelheid dan met de maximale bacterieconcentratie te verwachten. Het is daarom in het algemeen wenselijk bij zulk een bepaling de bacterieconcentratie op een relatief vroeg tijdstip, dus nog tijdens de groeifase, te bepalen.

Noer,B., 1961, Dansk Tidskr.Pharmac., **35**, 81

VIII

De experimenten, op grond waarvan *Juhr* tot een overleving van **Mycoplasma pulmonis** in water van 22 dagen concludeert, kunnen de toets der kritiek niet doorstaan.

Juhr,N.-C., 1971, Z.Vers.Tierk., **13**, 210

IX

Van sommige polypeptiden is aangetoond, dat zij onder katalytische invloed van Cu^{++} geoxideerd worden, en wel specifiek bij het derde aminozuur, gerekend van de terminale NH_2-groep. De speculatie die door *Levitzki et al.* ter verklaring is gelanceerd, lijkt minder waarschijnlijk dan de hypothese dat deze specificiteit het gevolg is van een trans-effect in het gevormde katalysator/substraat complex.

Levitzky,A.; Anbar, M., and Berger, A., 1967, Biochem., **6**, 3757

X

In het eerste echelon van de algemene gezondheidszorg, dient de patient omringd te worden door een team van deskundige hulpverleners van verschillende disciplines. Hierin zal de huisarts weliswaar de coördinator zijn, doch de verantwoordelijkheid delen met de overige leden van het team. In deze structuur zal het wenselijk zijn dat de discrepantie in opleidingsniveau en honorering tussen de huisarts en de overige teamgenoten wezenlijk wordt verminderd.

Ned. Huisarts. Genootsch., 1971, Conf. Driebergen, **71-34**, 18

XI

De vervanging, voor het wegverkeer, van de op aardoliederivaten lopende verbrandingsmotor door een andere krachtbron zou, hoe wenselijk dit ook moge zijn voor de vermindering van de luchtverontreiniging, grote problemen doen ontstaan voor de beschikbaarheid van bitumen voor de wegenbouw.

XII

De personeelsopbouw van laboratoria dient tijdig te worden aangepast aan de realiteit, dat steeds meer academici en steeds minder krachten met een middelbare vakopleiding ter beschikking zullen komen.

XIII

De verplichting om aan een thesis een aantal stellingen buiten het vakgebied toe te voegen, stamt uit een tijd waarin van de academicus nog een grote universaliteit kon worden verlangd. Het handhaven – in Nederland – van deze verplichting tot op de huidige dag, werkt de misvatting van gepromoveerden in de hand, dat hun opvatting ook over zaken buiten het vakgebied als gezaghebbend dient te worden beschouwd.

Ir.P.A.van Hemert

24 november 1971