(1) Notes on colloidon membranes for ultrfiltration and pressure dialysis / by G.S. Walpole. (2) Detection and concentration of antigens by ultrafiltration, pressure dialysis, etc., with special reference to diphtheria and tetanus toxins / by A.T. Glenny and G.S. Walpole.

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(1) NOTES ON COLLODION MEMBRANES FOR ULTRAFILTRATION AND PRESSURE DIALYSIS

BY

G. S. WALPOLE, D.Sc., F.I.C.

(2) DETECTION AND CONCENTRATION OF ANTIGENS BY ULTRAFILTRATION, PRESSURE DIALYSIS, ETC., WITH SPECIAL REFERENCE TO DIPHTHERIA AND TETANUS TOXINS

BY

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AND

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From

THE WELLCOME PHYSIOLOGICAL RESEARCH LABORATORIES

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BROCKWELL HALL

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XXVI. NOTES ON COLLODION MEMBRANES FOR ULTRAFILTRATION AND PRESSURE DIALYSIS.

By GEORGE STANLEY WALPOLE.

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The differential porosity of gel-membranes to molecules of different sizes may be utilised in a number of different ways all of which are essentially filtration. If the "filtrate" side of the membrane is bathed in any solution other than filtrate the process may be called "dialysis." Filtration under pressure is a very common procedure: but the many advantages to be obtained by dialysis under pressure have not received the study and recognition they undoubtedly deserve.

The difference between ultrafiltration and pressure dialysis.

Pressure dialysis brings about the complete removal of any constituent of the material handled whose molecules are by reason of their size and nature capable of traversing the membrane. Ultrafiltration, on the other hand, permits only the removal of those small molecules which can actually be brought into contact with the filter and pushed through. Hence, in order to separate a mixture of materials into those constituents whose molecules pass through a certain diaphragm and those whose molecules do not, either ultrafiltration or pressure dialysis may be employed. The latter process may be regarded as that which gives a quantitative separation, with the disadvantage that the materials passing through the membrane are recovered in a dilute condition, although the volume of residual solution is completely under control.

Collodion membranes. The use of membranes of collodion for the processes referred to will always be associated with the names of Rodewald and Kattein [1900], Malfitano [1904], Duclaux [1905, 1907], Moore and Roaf [1907, 1908], Lillie [1907] and Bechold [1907, 1, 2, 3; 1908]. The last author used pressure and introduced the use of formalised gelatin as a filtering medium. To him, too, we are indebted for the word "ultrafiltration," which has now passed into general use. In this matter C. J. Martin [1896] anticipated many later developments by fully describing and illustrating special apparatus by which solutions to be investigated were forced under pressure through films of gelatin or silicic acid. The material impregnated was a Pasteur-Chamberland filter candle, which served as a support against disruption. In conjunction with T. Cherry [1898] such filtrations were used to demonstrate that diphtheria toxin-antitoxin mixtures after two hours' standing at 30° gave toxin-free filtrates, although toxin when alone passed through the filters, and antitoxin, as shown by Brodie [1897] using Martin's method, did not.

My object in this paper is to draw attention to the possibility of standardising accurately a series of filters, suitable for "ultrafiltration" and pressure dialysis operations, which are already in use. Also, to call attention to certain members of the series, that is to say those containing 5 mg. of nitrocotton per sq. cm. and weighing 17·5 mg. per sq. cm. because of contained water. These have been discovered to be impermeable to all antigens tried, although they allow water, salts and simpler molecules to pass freely—a valuable conjunction of properties not shared, as far as I can discover, by the ultrafilters in general use.

To make the matter clear, it may be stated as a result of direct comparative experiments with parchment and from experience in the past with gelatin filters, that attempts to force diphtheria toxin under pressure of two atmospheres through parchment or gelatin would not result in nearly so much filtrate as if one of the "antigen-proof" collodion films to which I have referred were used. Further, as may be seen by referring to the original papers from the references given, they would not be found to keep back antigen to any great extent.

The reason of this must, of course, in the present state of our knowledge of gel structure and the phenomena of filtration generally, be given with reservation. It would appear, however, not an unreasonable suggestion that these films possess an enhanced uniformity of structure and a great porosity. In other words, the channels leading from one side of the filter

to the other are none of them sufficiently large to let the antigen pass, but there are many of them. On the other hand, it is suggested that in parchment, for example, these holes are of irregular size. Some are large enough to let diphtheria toxin through, but the total amount of water they will allow to pass is, in virtue of the lower general porosity of the structure, less in this case [Glenny and Walpole, 1915].

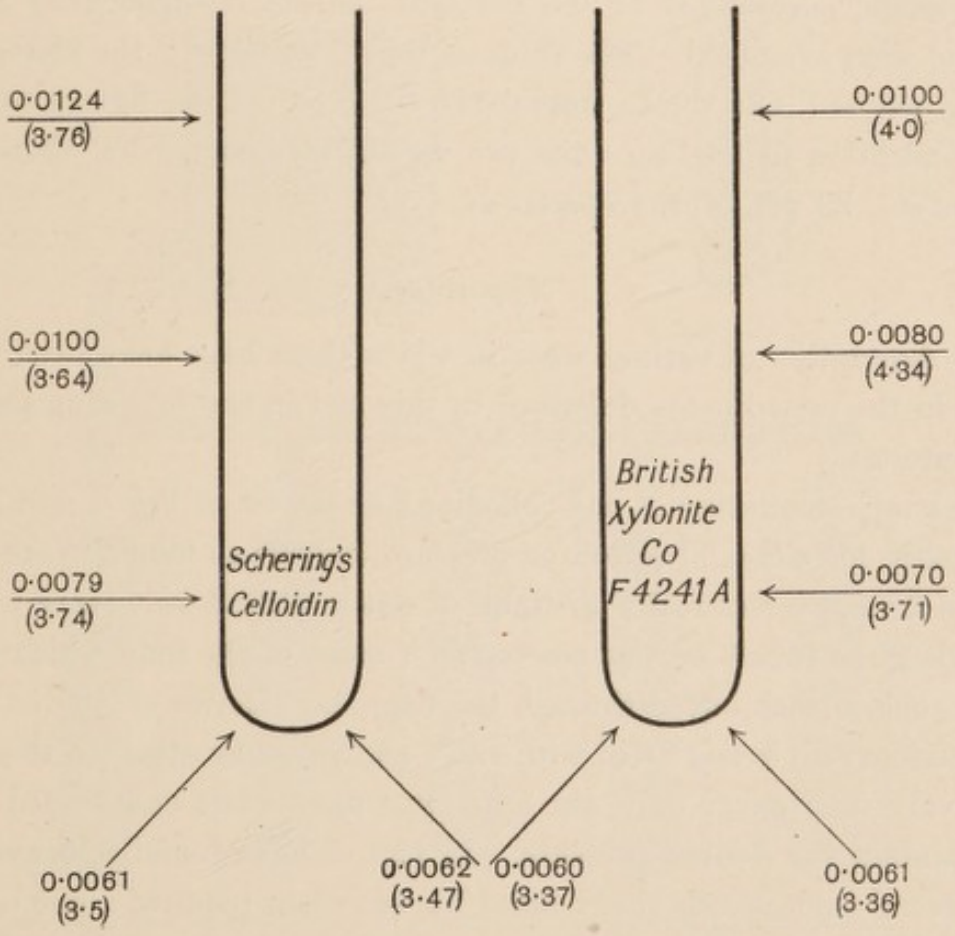
Collodion used. As a stock solution a 14 per cent. solution by weight of Schering's celloidin in equal parts of alcohol and ether was originally employed. Later the British Xylonite Company have kindly supplied me with a similar material ready dissolved and filtered clear. It is named for reference F 4241 A and supplied by them in tins with convenient screwed stoppers at a price which represents a considerable saving in expense. The solutions when used in the same manner give very similar results as may be judged from the comparative experiments given below (page 287).

The preparation of collodion bags. These have been often described by Malfitano [1904], Bigelow [1907] and many other authors. That they may be made with differences of porosity is generally known. For information about them and instructions how to make them I am indebted to Prof. S. P. L. Sörensen and Dr Christiansen. They are sometimes made by pouring consecutive layers of the solution on to the outside of a test tube rotated by a suitable mechanism. Probably the bags made in this way are more perfect than those made inside test tubes, but special apparatus is required and, I should judge, considerable experience and skill in order to be sure of producing a bag of just the required impermeability. The following is a description of the bags which I have adopted as "standard," though for anything beyond odd quantitative experiments I prefer to use the flat sheets described in some detail later, for they possess certain advantages over any cylindrical bag however perfect.

A test tube is filled with "stock solution," capped with tin foil and after the twelve or fifteen hours required for the bubbles to rise is inverted and clamped so that the mouth is well above the wide mouthed stock bottle. When the flow of collodion ceases the bottle is removed and stoppered and about ten minutes later the drippings are cut away from the test tube with a sharp knife, care being taken not to pull loose the lower edge of its collodion lining. The test tube must, of course, be cut off sharp and not lipped or opened at the mouth in any way. Drying is allowed to continue until the inside when rubbed gently with the finger does not mark at all—a degree which cannot be described precisely—a process taking about half an hour at

laboratory temperature. The test tube and its invisible collodion lining are now placed under water, and at a later time while still under water the lining may be easily removed and tied to a rubber stopper. It is convenient to remember that these membranes shrink rapidly in air and become less porous, though in cold water they may be stored indefinitely. In hot water they shrink, but may be stretched and moulded without undergoing any apparent permanent change—a property which makes it possible to mount them on rubber stoppers of slightly larger diameter than would otherwise be possible.

Some simple measurements of two bags, made at the same time, one from Schering's celloidin (13·45 per cent. by weight in equal parts dry alcohol and ether) and the other from British Xylonite Company material, F 4241 A (14·66 per cent. in the same solvent), may be of interest. The unavoidable lack of uniformity of bags made in this way is apparent from the diagram below, in which the figures represent the weight per sq. cm. of dry nitrocotton and the "wetness" at the points indicated (see page 292). The latter are in brackets. The dimensions of these bags are 4·5 cm. wide and 22 cm. long.



Smaller bags than this, even though made with great care, are still less uniform. Three other bags from each material, made at the same time under conditions as similar as it was possible to arrange, were tested. One pair filtered diphtheria toxin, a second pair filtered tetanus toxin, while a third pair were tested by pressure. They both ruptured between two and three atmospheres pressure after stretching considerably.

Ultrafiltration of tetanus toxin. Batch Z. Both bags were fed with toxin at 20 cm. of mercury pressure for about a week; the Schering's celloidin bag passed 2600 cc., while that made from the British Xylonite Company's material passed 2300 cc. The former filtrate gave a very faint indication of toxicity: none could be detected in the latter.

Ultrafiltration of diphtheria toxin (mixture of equal parts J 2133 and J 2159, L_+ dose 0·40 cc.: nitrogen = 5·3 mg. per cc.). The pressure applied equally to both bags was 20 cm. of mercury for twelve days. The bag from Schering's celloidin allowed 1875 cc. to pass; that from British Xylonite Company material filtered 2155 cc. No toxin could be detected in the latter filtrate: 1 cc. of the former gave a slight swelling in the guinea pig.

It may be remarked in passing that the residues in the bags were, at this juncture, surrounded by 0·3 per cent. phenol changed daily for three days and then examined. The volumes were 100, 85 cc.; the nitrogen 2·83, 2·66 mg. per cc.; and the L_+ dose 0·022, 0·018 cc. These figures correspond to concentration of binding units per mg. of nitrogen of 33, 43 times and yields of 81, 89 per cent. respectively.

Apparatus.

The description of various ways in which these bags have been mounted for use in the experiments described in this and in the following paper may be of interest.

The simple mounting of one collodion bag shown in Fig. 1 will be found satisfactory for either filtration or pressure dialysis as long as care is taken to make the apparatus really air-tight, or else to attend to it every few hours. The wide glass tube *b* acts as reservoir for some of the fluid which will ultimately replace that forced through the bag, and also for air under pressure. If an auxiliary air vessel fitted with valve and gauge be attached to *a*, instead of the valve and gauge only, the apparatus can be left unattended for days and maintain the desired pressure. In fact, I have found it convenient to have several such vessels ready fitted for use when required: one of them is shown in Fig. 3 (p. 290). By means of a branched tube it can, of course,

Fig. 1.

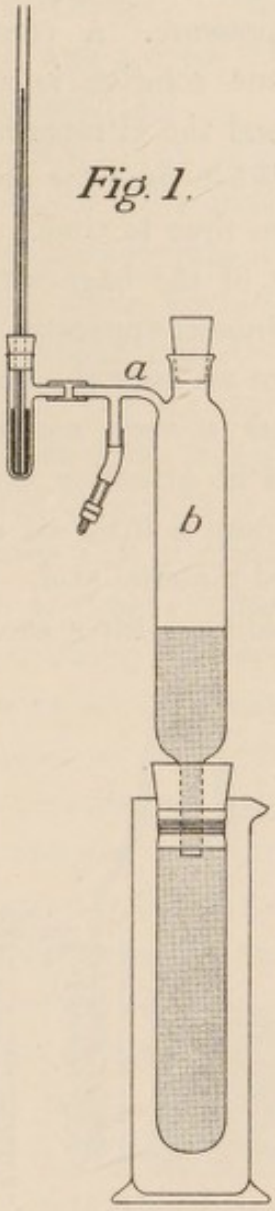


Fig. 2.

supply several pieces of apparatus like that sketched in Fig. 1 with air at the same pressure. A convenient mounting for five such bags all holding the same solution is shown in Fig. 2—they are all fed at the same pressure and the filtrates may be compared and so differences in bags investigated. When used for pressure dialysis the water in the jars must be changed from time to time. The inconvenience of not being able to get at the contents of the bags without dismounting the apparatus naturally suggests a continuous apparatus such as that in Fig. 3 for pressure dialysis. The arrangement whereby the toxin and 0·3 per cent. phenol flow in opposite directions effects a very considerable economy in distilled water. The apparatus needs no attention except daily to fill the aspirator with 0·3 per cent. phenol, pump a little air into the reservoir and run off the yield of pressure-dialysed material at *d*. The bottles indicated on the left are double Winchesters holding 5 litres each.

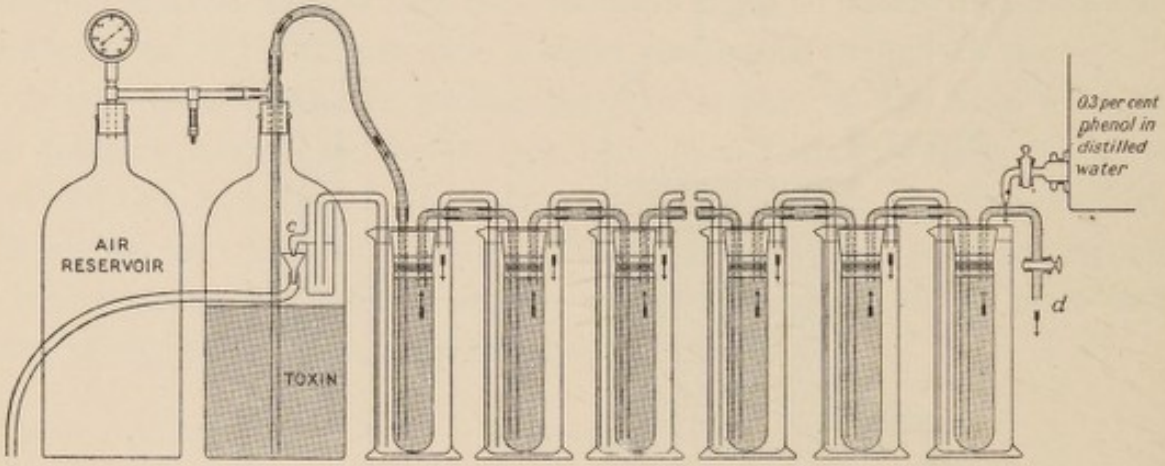


Fig. 3.

The overflow at *c* empties direct to the drains. In two sets of this kind the process has gone on for upwards of three months, in each case without any signs of the bags becoming choked. Apart from the economy of bags, for a set of eight have handled 100 litres of toxin and are still in use, there is possibly the additional advantage that the loss due to adsorption in the bag is minimal, since the volume of toxin handled by each bag is so great. Further details of the working of this apparatus are given in the following paper [Glenny and Walpole, 1915, p. 301].

To avoid the clumsiness of large scale operations in glass apparatus, and the limitation of pressure to that which unsupported bags will stand, it is an obvious step to transfer these operations to properly designed metal

apparatus in which use is made of flat membranes. These, unlike the collodion bags described, are uniform all over and possess the advantages of being capable of accurate calibration and reproduction.

The preparation of flat collodion sheets. Although in practical concentrations a great deal can be done without the use of flat membranes, for a bag can always be made on the "safe" side with a corresponding loss of rapidity of filtration and other properties of more or less value, nevertheless the latter have many advantages. For large scale technical operations impregnated cloths offer greater permanence and strength, but for research work and technical operations on the smaller scale, where there is opportunity to use a reasonable amount of care with the resulting films, I have taken advantage of the increased simplicity of films of water and nitrocotton only. The absence of supporting material such as cloth or canvas makes it much easier to study the properties of these systems accurately, to reproduce them at will and to arrive at a nice discrimination as to the most suitable membrane for any particular purpose. If a thin membrane of high porosity and therefore low tensile strength be advisable for a particular filtration, I suggest that a fine nickel wire gauze or a cloth to support it should be placed not in the membrane but behind it. The homogeneity of the membrane is then not disturbed and measurements such as thickness, tensile strength, permeability to water, water content, etc., bear results which are more intimately applicable to a rational study of the problem in hand.

A square or round sheet of plate glass larger than the finished filter required is levelled accurately, and on to it is poured a measured quantity of "stock" solution suitably diluted with a mixture of equal parts of dry alcohol and ether. Care should be taken that the collodion solution is poured on to the centre of the glass, and that it is sufficiently diluted to run right to the edge. As it will not run over unless an unnecessarily large volume be taken, the glass really acts as a very shallow dish. As evaporation proceeds, the film slowly hardens until it is decided to transfer the glass plate with its attached film to water, beneath which they are separated after the lapse of a few minutes by one steady pull. At any time later a filter the exact size required is cut out of the film with a template and a knife. A good commercial half-inch plate glass is cheap and quite flat enough to give films, say eight inches across, differing between their thickest and thinnest parts by only 40 μ or less. To obtain such films I have taken pieces of glass 12 inches square. Square films will be found thinner at the corners than at the centres of the sides if the volume of collodion poured be insufficient.

A tendency which sometimes occurs to curl up and leave the glass in dry or warm weather may be prevented by painting the edges of the hardening film with "Collodion Flexible B.P."

Now the degree and temperature of nitration determine the physical properties of nitrated cotton to a most marked extent, so that the properties of the film ultimately obtained depend upon a host of factors, of varying degrees of importance, starting with the manner of nitration of the cotton and ending with the pressure applied to it in use. To characterise membranes therefore so that they may be reproduced and their individual properties discussed, the only possible plan is to take in every case a quantitative account of the result of all variables in procedure which are recognised and utilised as such, and by using stock solutions either identical or as similar as possible, and constant conditions, to reduce the other factors as far as is experimentally convenient to constancy.

The variables I have utilised are two in number and both of them are readily controlled and expressed numerically. The first is the weight of dry nitrocotton per sq. cm. (m) of the finished film. This is determined beforehand, for the strength and volume of the solution are known as is also the area of the plate over which it is poured. The second is the "wetness" of the resulting film, a property, intimately connected with its porosity, which depends primarily upon the amount of solvent still remaining in the nitrocotton on the glass plate before it is plunged into water. As a numerical expression of the "wetness" (w) of a filter I have found it convenient to use the ratio of its weight to that of the dry nitrocotton it contains.

For example, 157 cc. of a 3·5 times diluted "stock" solution of Schering's celloidin (13·5 per cent. by weight) were poured on to a circular plate 30·5 cm. diameter.

Hence $$m = 0{\cdot}0082 \text{ g.}$$

The weight of a circular filter 23·5 cm. across cut out of this sheet was 17·5 g. The weight of dry nitrocotton it contains is therefore $\frac{0{\cdot}0082 \times \pi \times 23{\cdot}5^2}{4} = 3{\cdot}56$ g.

Hence $$w = \frac{17{\cdot}5}{3{\cdot}56} = 4{\cdot}91.$$

Direct determinations, by weighing, on small pieces from the remainder of the film after the circular filter had been cut out gave

$$m = 0{\cdot}0081 \text{ g.}$$
$$w = 5{\cdot}02.$$

Now, to test the uniformity of a film it is often convenient to make a few measurements of thickness (t) with a micrometer. The formula connecting m, t and w is found, in practice, to be that which follows at once from the assumption that the thickness of the film is the sum of the thicknesses of the nitrocotton and water in it. The density of nitrocotton may be taken as 2.

$$t = m\left(w - 1 + \frac{1}{\Delta}\right) = m\,(w - 0{\cdot}50).$$

Hence, when making filters to specification it is more convenient not to use the balance at all, but to use a micrometer not only for testing the uniformity of the finished filter, but also for the purpose of determining the correct drying time for any particular film. A thin strip from any part of the film which is just clear of that part which will be required for the finished filter is cut on the glass, peeled off, plunged into water and examined with a micrometer a few minutes later. The thickness gives at once information as to how far the drying has progressed.

Two experimental films, the drying of which was followed in this way, furnished the examples given below:

Example 1. A 13·5 per cent. solution by weight of Schering's celloidin had been diluted 4·5 times. Of this 150 cc. were poured on to a plate 30·5 cm. square, and strips were cut from time to time. Each was immediately after removal from the glass plunged into water, and its thickness measured as the mean of five readings at equidistant points on that part of the strip which would be included in a filter 20·5 cm. square. Direct determinations of m and w were made by weighing pieces of known area both wet and after drying to constant weight. The temperature of the room was 10°–12°.

Example 2. A film containing more nitrocotton per sq. cm. was made by pouring 200 cc. of the "stock" solution above after 3·5 times dilution on to a square plate of the same size. Temperature, 12°–14°.

	Hours of drying before strip was pulled off plate	Mean thickness, t, after immersion in water cm.	Maximum variation from mean thickness μ	m by weighing pieces of known area dried to constant weight	w by weighing pieces wet and after drying to constant weight	w calculated from formula $t = m \times (w - 0{\cdot}5)$
Example 1	2	0·0435	−5	0·00488	9·28	9·41
	3	0·0322	+3	0·00493	6·97	7·03
	4	0·0182	−12	0·00515	4·00	4·03
	5	0·0110	0	0·00503	2·63	2·68
	6	0·0094	−4	0·00512	2·29	2·33
Example 2	2·25	0·0587	+17	—	7·16	—
	3	0·0484	+16	0·00717	7·29	7·25
	4	0·0337	+13	0·00679	5·48	5·46
	5	0·0194	+13	0·00635	3·56	3·55
	6	0·0117	+3	0·00684	2·15	2·21

Press for the use of flat sheets. Fortunately these collodion-water membranes make excellent joints, so that the construction of a press (Fig. 4) to take them without leaking is a comparatively easy matter, and as there is no precipitate to be dealt with, the frames may be thin and narrow and the channels small. Instead of horizontal channels leading from frame to frame I have drilled the frames and soldered in nipples over which suitable rubber tube may be passed. Such a connection between any one nipple and another is instantly made and can be relied upon to stand with certainty for an indefinite period pressures higher than those which I have found it convenient to use. A press permits the employment of pressures which an unarmoured bag could not be expected to withstand, and, constructed in this way, is without prejudice as to the use to which it may be put—whether a periodic or a continuous process—whether filtration, dialysis, or a combination of the two. This is advisable, for such a press as this may well be called upon to do the routine concentration of diphtheria toxin (a continuous pressure dialysis process), that of tetanus toxin (a periodic process—ultrafiltration followed by pressure dialysis), to free tuberculin from glycerol on occasion, and to do any experiments of a similar nature that may be required.

No plates are used in the press—between each pair of membranes and the next on either side are inserted perforated and corrugated material when the press is made up. The celluloid sheet used for keeping apart the lead plates of certain types of accumulators is eminently suitable for the purpose when only low pressures are used. Care must, of course, be taken in filling the press to see that the membranes are not burst by applying pressure before it is full. Although I have done without them in this apparatus, plates with a flat edge but embossed elsewhere with a suitable pyramid pattern would undoubtedly give the advantage of additional security in this respect.

In another apparatus, not illustrated, which I have used as an ultrafilter only, one such plate, ten inches across, covered on each side with a membrane and placed between two flat half-inch steel plates has been used with success. Liquid to be filtered is forced through fine passages in both outer plates and the filtrate runs out from a small hole in the side of that between. The filtering area is 831 sq. cm. and the amount of fluid lost in the apparatus is 2–3 cc., e.g. 50 cc. of blood serum at 2 atmospheres gave in 20 minutes 40 cc. of protein-free filtrate containing sugar and salts but giving no biuret reaction. The residue recovered on gently aspirating at the inlet tubes was 7·5 cc. The filters used were toxin proof, but for the particular experiment quoted much softer and more porous films would have done.

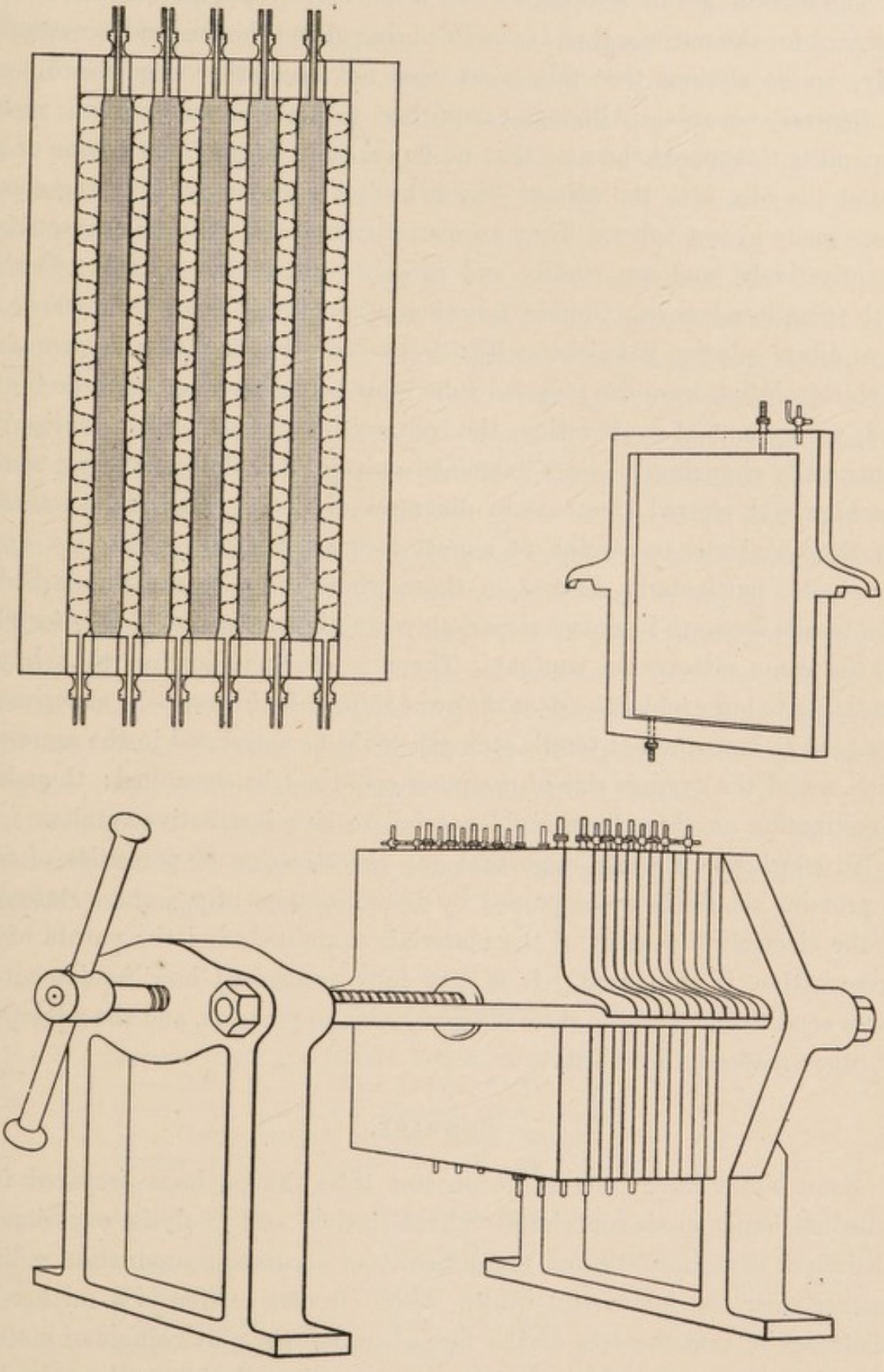


Fig. 4.

The advantages of working in this fashion with apparatus more suitably designed for the purpose than is possible using glass tubes and rubber stoppers only, are so obvious that this point need not be further emphasised.

General remarks. Although requiring a certain amount of special apparatus to support them so that no liquid to be filtered can escape or get round the film into the filtrate, the flat films offer many advantages over those made in test tubes. They are perfectly uniform, may be characterised quantitatively, and are readily and exactly reproducible at will. Dealing with them involves less trouble, for when a flat film is poured the 200 cc. or so of dilute solution is finished with: but when a bag is made the droppings of thick solution from the inverted tube must be collected again in the bottle, and, on account of evaporation, the composition of the "stock solution" is continually changing. I have examined strips of flat films in a paper testing machine and plotted stress-strain diagrams of them. Their liquid nature, for they continue to stretch at almost constant tension, is, as one would anticipate, particularly marked in those whose main constituent is water. The tensile strength increases remarkably rapidly as "wetness" falls for films of the same nitrocotton content. There is every indication that impermeability to large molecules does the same thing. Unfortunately, experiments designed to test whether tensile strength could be correlated in the same way with m and the average size of the pores could not be continued: though an investigation on these lines could not fail to give instructive results.

In conclusion I would urge that any investigation of porosities of films to proteins should be accompanied by determinations of p_{H^+}, and a statement of the electrolyte content of the materials examined, and the nature of the preservative, if any, used. It is now fully recognised how fundamentally these conditions affect the state of aggregation of proteins, and the adsorption of other materials by them.

Summary.

Some notes have been made on test tube shaped bags prepared from collodion, and used for simple ultrafiltration and dialysis experiments. Their lack of uniformity and the difficulty of accurate reproduction militate against their use for careful work. Their circular section is a further disadvantage in that the area of the film operative per unit volume of material treated is large only when the tubes are small, and that, unless the bags be armoured, the pressure that can be used is limited to that which the unsupported membrane can withstand.

Flat membranes may be made by pouring alcohol-ether solutions of collodion on to levelled plate glass, and then plunging the film, from which alcohol and ether are more or less removed by evaporation, into water. Using this method, and taking certain precautions described, they may be made with remarkable uniformity. By keeping as far as possible to constant conditions, these membranes may be described fully, for purposes of reference and reproduction and as a guide to their properties, by stating the kind of nitrocotton used, the weight of it per sq. cm. they contain, and the "wetness," i.e. the ratio of the weight of a piece of film to its weight when dried to constancy. Films, made in the manner described, containing 8 mg. per sq. cm. of the particular nitrocotton used and weighing, with contained water, 40 mg. per sq. cm., or others where $m = 5$ and $w = 17{\cdot}5$, have been found to retain quantitatively all antigens with which experiments have been made. Practical applications of this result are given in the paper following this [Glenny and Walpole, 1915]. Their permeability to simpler molecules, salts, and water remains high, and filtration through them is rapid.

It is suggested that in these films, more than in other ultrafilters in general use, the channels leading through the gel structure from one side to the other are of a certain uniformity in size. None of them is large enough to allow any antigen to pass; but the structure is highly porous and, because there are many such channels, rapid ultrafiltration results.

Arrangements of apparatus are described whereby films of this type whether flat or in the shape of test tubes may be made and utilised for ultrafiltration and pressure dialysis.

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XXVII. DETECTION AND CONCENTRATION OF ANTIGENS BY ULTRAFILTRATION, PRESSURE DIALYSIS, ETC. WITH SPECIAL REFERENCE TO DIPHTHERIA AND TETANUS TOXINS.

By ALEXANDER THOMAS GLENNY and GEORGE STANLEY WALPOLE[1].

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(*Received May 21st, 1915.*)

One has but to know something of the precarious character of routine toxin production to be aware that the artificial conversion of low value toxins to those of high titre is a problem of more than mere academic interest.

In the search for a suitable process by which this may be done it is soon realised that purely chemical methods such as those referred to so completely by Pick [1912] are not available, as they are usually cumbersome and costly, and any effective concentration invariably entails an enormous loss of antigen. Amongst other possibilities the ammonium sulphate method of Heinemann [1908] failed in my hands, possibly because phenol was present; while an alcohol method, applied by Banzhaf, I have not tried on account of expense, for it is essential that any routine process should be simple and cheap. The matter was left at this stage for several years until the accidental discovery was made that on acidification diphtheria toxin, from which salts had been removed by dialysis in a collodion membrane, developed a slight precipitate which contained all the toxin. Moreover, collodion membranes of a special kind [Walpole, 1915] could be made which, though reasonably permeable to water, salts, etc., nevertheless retained quantitatively diphtheria toxin and

[1] A. T. Glenny is responsible for all the tests referring to diphtheria and tetanus toxin purification which require the use of animals; and, also, for the study of the question from the immunological standpoint. The details of physico-chemical technique are supplied by G. S. Walpole.

all other antigens with which experiments were subsequently tried. These unexpected results, in flat contradiction to those of Baroni [1911] for example, who found diphtheria and tetanus toxins to pass through the collodion membranes he employed, form the basis of the concentration methods here put forward. The various experimental difficulties have disappeared as the result of experience, but, so far, it is only in the case of diphtheria toxin that precipitation by a trace of acid after the removal of the salt can be used as an effective short cut to a purification of the antigen. The invariable result, so far as my experience goes, that antigens do not pass through these membranes suggests their employment not only for routine toxin purification, but in addition as a laboratory test to form a first opinion on the specificity or otherwise of the toxic constituents of any cultural fluid.

The Purification of Diphtheria Toxin.

A batch of diphtheria toxin is appraised by the immunologist by its "binding unit" content per cc., a quantity which for all purposes is most readily measured as the L_+ dose, though this would not necessarily be so if the original media were not similar or had not disintegrated, during the growth of the bacillus, on similar lines. Also, the reservation is made that the "binding units per cc." of these broths are by no means *proportional* to their utility: rather some such scale as the following is taken:

L_+ dose	Use for immunising
0·15 cc.	"excellent"
0·30 cc.	"good"
0·50 cc. and over	"unusable."

In other words poor toxin cannot be made to act as good toxin simply by taking more of it, for experience has shown that the development of antitoxin in the horse is greater when the effective immunising material is interfered with in its action as little as possible by accompanying nitrogenous matter.

It may therefore be taken as a working hypothesis, remembering that the experience of the immunologist is the final and only appeal, that, providing the loss of toxin is minimal, the extent to which the binding units per milligram of nitrogen is increased by this purification process is some sort of measure of its efficiency. When the material accompanying the toxin is so far reduced as to be physiologically of no importance in the dose accompanying the greatest toxin injection given, it is obviously not a true measure because

further purification serves no useful purpose: it may be inadvisable, in fact, for one would suspect, on the ground of general experience, an increased instability of the toxin as it approaches purity.

The essential process we have employed to concentrate and purify diphtheria toxin is to dialyse it under pressure using a collodion membrane of a particular kind dealt with more fully, for the sake of convenience, in a separate paper [Walpole, 1915]. The dialysed material is then acidified and centrifuged. The precipitate, dissolved in a trace of alkali, constitutes the concentrated material. It should be kept in the cold after the addition of a small quantity, say, 0·3 per cent., of phenol.

To know when the material has dialysed sufficiently to pass on to acidification either of the following methods may be used. A sample may be tested by adding 0·3 cc. N acetic acid to 10 cc. If the precipitate formed flocculates well and leaves a bright supernatant fluid no further dialysis is necessary. This point corresponds, for the broths which have been dealt with, to that where no more colouring matter passes out through the walls of the bag and to a conductivity of about 0·00078, i.e. that of 0·0065 N KCl. For routine work with a continuous apparatus [Walpole, 1915, p. 290] rough conductivity determinations are much the most convenient method for checking the working and determining how much dialysed toxin may be drawn off each day in safety.

Results.

Where this process is applied the cost of toxin production is considerably reduced, for batches are always worth the units they contain, whether they are good or bad, and no toxin however poor need be thrown away. Periodic recurrences of bad toxin to which every laboratory is subjected need, therefore, no longer be feared.

The following generalisations summarise the results of our experience upon which this concentration process for diphtheria toxin had been developed.

1. *Whether by pressure dialysis or ultrafiltration no toxin passes through these membranes.*

These results have been checked so many times that special experiments need not be cited. Part of the evidence supporting them will be found in the ensuing pages. [See also Walpole, 1915, p. 288.]

As a matter of convenience, however, the details of some routine concentrations are given at this juncture, for they depend essentially upon the impermeability of these bags to diphtheria toxin.

At the time of writing two sets of apparatus [Walpole, 1915, Fig. 3] each consisting of eight bags which were set up some months ago are still in use. One has handled over 100 litres; the other about 44 litres, of which two small batches were worked out more or less completely.

Details of concentration experiments. March 12th to April 1st. Using the continuous apparatus [Walpole, 1915, Fig. 3], 12 litres of diphtheria bouillon, J 2520 (L_+ dose = 0·40 cc.: nitrogen = 2·8 mg. per cc.) gave, in 20 days, 8500 cc. of pressure dialysed material (nitrogen = 1·96 mg. per cc.). The process was not hurried in any way; nothing was drawn off for three days, then 500 cc. per day were collected through the run, and then a couple of days allowed to elapse before all the bags were emptied. The excess of pressure used was 0·3 atmosphere and the consumption of the dialysing fluid, 0·30 per cent. phenol in distilled water, ten litres per day. After acidification the precipitate, representing twelve litres of the original bouillon, was dissolved in alkali and diluted to 250 cc., giving a solution whose L_+ dose was found to be 0·012 cc., and the nitrogen per cc. 2·80 mg.

The result may be roughly stated that, with a loss of 30 per cent., the binding units per mg. nitrogen and also the binding units per cc., have been increased 33 times.

The previous batch of toxin treated by this set of collodion bags was J 2530, of which 11·5 litres (L_+ dose 0·40 cc.: nitrogen 4·6 mg. per cc.) gave 230 cc. of concentrated material (L_+ dose 0·01 cc.: nitrogen 6 mg. per cc.) representing a yield of 80 per cent.

When the same set of bags had handled 50 litres of material whose L_+ dose was 0·4 cc., a sample of dialysate from *C* was collected. They were then lifted clear from the glass jars containing them and some of the ultrafiltrate dripping from them collected (that collected in the first hour or two was, of course, discarded). Neither of these solutions interfered with the increase of weight of the guinea pig when injected in a dose of 1 cc.

The lost toxin, therefore, does not pass through the bags, nor is it to be found in the supernatant fluid above the precipitate produced by acidification. It would seem probable that it represents destruction by light, or by being left at room temperature and in a state of increased purity at a reaction which is probably not that at which it is most stable, at some stage in the process. Experiments on this point are in progress. Should the loss be actually in the filter itself the recovery therefrom of a highly toxic material may be anticipated.

2. *The toxin goes through the best and thickest parchment commercially obtainable, though the parchment is far less permeable to water than the collodion membrane through which toxin cannot pass.*

A large volume of toxin J 2536 (L_+ dose = 0·35 cc.) filtered through parchment under a slight pressure yielded after some days 50 cc. of filtrate resembling the original in colour and general precipitation reactions. The L_+ dose of the filtrate was 0·60 cc. This is a striking example of the fact that low permeability of a membrane to water is not necessarily accompanied by low permeability to toxin. Using the same batch of toxin and a collodion membrane ($m = 5$: $w = 3·5$, [see Walpole, 1915]) no toxin could be detected in the filtrate (1 cc. nil), though the volume of the filtrate per square centimetre per hour was many times greater in this case. Below are given details of a repetition of this experiment, using another sample of the parchment and another collodion bag.

Collodion bag.	Parchment bag.
Area exposed 260 sq. cm. Time 24 hours. Filtrate 8 cc., of which 1 cc. has no effect on the guinea pig. Pressure 6″ water.	Area exposed 1960 sq. cm. Time 24 hours. Filtrate 6 cc. of which 0·1 cc. kills a guinea pig. Pressure 7″ water.

This point is, in our opinion, worth full and patient investigation because of its far-reaching consequences in bacteriological work generally.

3. *The amount of toxin in a cultural filtrate bears no relation to the amount of precipitate obtained on acidification after pressure dialysis through these membranes.*

4. *The method is universally applicable at least to all brews of diphtheria toxin of the type made in these laboratories.*

Samples of 200 cc. each from twenty-nine different brews of toxin were dialysed, each in a separate bag, under pressure for four days against distilled water changed daily. Colouring matter had then ceased to come out through the bags and the specific conductivity of the inside fluid was in every case less than 78×10^{-5}. All gave nicely flocculated precipitates on adding 6 cc. of 1·0 N acetic acid to the contents of each bag. They were placed in order of the amounts of their precipitates as well as could be judged by eye. The first fifteen precipitates were then mixed and dissolved in 60 cc. of distilled water. Equal volumes of the corresponding original brews were also mixed. Both solutions were tested for "binding units" with the following results.

The first fifteen samples amounted in volume to 3000 cc. (L_+ dose = 0·45), from which were obtained 60 cc. of concentrated material (L_+ dose = 0·012).

The results referring to the remaining fourteen samples which all gave smaller precipitates when treated in the same way may be summarised:

2800 cc. (L_+ dose = 0·45) gave 56 cc. (L_+ dose = 0·012).

Though no other attempts were made solely to settle these points they are confirmed by the general experience of numerous other concentrations. The yield in both the above cases is 80 per cent., and the concentrations of the binding units per unit volume fifty times.

5. *If the cultural filtrate after pressure dialysis is only partially precipitated by a trace of acid, and if, after removing this small precipitate, it is completely freed from precipitable matter by further acidification, then nearly all the toxin will be found in the first precipitate.*

Four litres of a mixture of toxins (L_+ dose = 0·40: nitrogen 4·63 mg. per cc.) gave as first fraction material which, dissolved in a trace of alkali, was diluted to 80 cc. The L_+ dose was 0·008 cc. and the nitrogen content 3·5 mg. per cc. in this solution. The second fraction, comprising the rest of the precipitable material, when similarly treated gave 80 cc. of a solution of L_+ dose 0·30 cc. and nitrogen content 6·02 mg. per cc. Hence the yield of binding units on the first precipitate is as near as one can tell quantitative, and that on the second, which contains nearly twice as much nitrogen, is only 2 per cent. of the total binding units originally taken.

This experiment was performed either to confirm or throw doubt upon a previous result in which the distinction between the binding unit content per mg. nitrogen of successive fractions was equally marked.

6. *There is no evidence that this routine concentration process brings about any separation of the toxic from the binding elements of the cultural filtrate.*

The following two experiments were made with a batch of toxin J 2538, the L_+ dose and M.L.D. of which were 0·38 cc. and 0·005 cc. respectively. In both of them the concentrations of L_+ dose and M.L.D. were, within the experimental error, equal.

Experiment 1. 200 cc. of original toxin yielded a trace of precipitate which was dissolved in a convenient volume, which amounted to 16·6 cc. The L_+ dose of this solution was 0·05 cc. and M.L.D. 0·0007 cc. It is seen that both the toxicity and the binding units have been concentrated to about the same extent, i.e. 7·1 times in the one case and 7·6 times in the other.

Experiment 2. 1800 cc. of original toxin yielded 28 cc. of concentrated toxin having L_+ dose 0·008 cc. and M.L.D. 0·0001 cc. The concentrations are, in this case, forty-eight times for the L_+ dose and fifty times for the toxicity.

7. *Some change occurs in the material undergoing pressure dialysis which is not reversible, for it is impossible to reconstruct the original material by adding to the fluid remaining in the bag a corresponding amount of dialysate after concentration to the correct volume.*

The original material gives no precipitate on acidification: the reconstructed material does, i.e. it appears as if the salt when once removed fails to inhibit precipitation by acid when again added.

500 cc. of diphtheria toxin, brew Y 76, gave 5 litres of pressure dialysate (A) against 0·3 per cent. phenol, with a reduction in volume to 180 cc. (B). Comparative tests showed that the addition of 2 per cent. sodium chloride reduced by only very little the precipitate obtained by acidification. Similarly, if 170 cc. of A concentrated ten times by evaporation be added to 9 cc. of B, almost a full precipitation still occurs.

The immunising value of concentrated diphtheria toxin.

In the light of experience it is easy to establish whether any particular toxin has good immunising value or not, but to demonstrate statistically the comparative values of two toxins would require many horses owing to their individual variations.

Two normal horses immunised with concentrated toxin prepared from material so poor in value as to be unusable reached 800 A.T. units and 1400 A.T. units per cc. Individual horses immunised with unconcentrated toxin have reached 2000 A.T. units per cc., but it requires more than usually good toxin to reach an antitoxic unit content of even 1400 per cc. The average value of horses under similar treatment with good unconcentrated toxin was 700 units per cc.

The details of the response to treatment, although referring to two horses only, indicate clearly that concentrated toxin is at least as good as unconcentrated toxin unit for unit for purposes of immunisation. The volume of the injections was very small, though the number of binding units injected was the same as that usually given when unconcentrated toxin was used. The general condition of both the horses was far better than that of horses immunised in the ordinary way.

Another horse that had received several immunisations of unconcentrated toxin was gradually falling off in unit value. After a course of concentrated toxin, in which the largest dose given was 10 cc., higher value sera were obtained from it than had been obtained at any time during the previous treatment.

These experiments on the immunising value of concentrated diphtheria toxin, as compared with that not so concentrated, are being continued.

Purification of Tetanus Toxin.

Owing to the vigorous digestion of the broth constituents by the tetanus organism the concentration of toxin per mg. nitrogen obtained by ultrafiltration followed by pressure dialysis is in this case greater even than in that of diphtheria cultural filtrates. The rapidity of filtration is, too, for the same reason, much greater. The slight precipitate obtained on acidifying the dialysed material is, as has been already stated, not toxic. This curious difference between diphtheria and tetanus toxins must be of considerable theoretical significance.

In the tests given below the animal used was the mouse.

Experiment 1. An experiment has been described [Walpole, 1915, p. 288] where comparative tests of the porosities of two bags showed that no toxin could be detected in one of the filtrates and very little in the other. A dose of 0·0005 cc. of the original material placed in the bag caused death on the 4th day.

Experiment 2. Toxin BDC (0·0005 cc. death on 4th day); 30 cc. were concentrated to one-seventh the volume with continual change of water outside, and diluted again to its original volume: no change in M.L.D. could be detected. A small precipitate obtained by acidifying and centrifuging an aliquot portion was found to be non-toxic.

Experiment 3. 190 cc. of toxin BDC were reduced to 6 cc. as above. Of this

0·00005 cc. gave death on the 3rd day
0·00002 cc. ,, ,, 7th ,,

that is to say, that the toxin was concentrated about twenty times and the volume reduced to one-thirtieth.

Experiment 4. Toxin BDA (L_+ dose 0·05 cc.); 10 cc. of this were reduced to about 3 cc. and then made up to the original volume with physiological saline solution. The L_+ dose was found to be unaltered.

Experiment 5. 10 litres toxin BKA (1 cc. contains 3·7 mg. nitrogen) gave in the apparatus described by Walpole [1915, Fig. 2] in six days 9630 cc. of ultrafiltrate divided between five bags. Tests on these filtrates showed that one bag only allowed a detectable amount of toxin to pass through (M.L.D. 0·4 cc.). Twenty-four hours' pressure dialysis followed, and then

300 cc. of concentrated and purified toxin containing 3 mg. of nitrogen per cc. were collected from inside the bags.

It would seem from these observations that the horse, while still receiving the full measure of specific antigen, may be spared 97 per cent. of the nitrogen and, in this case all the indole, fatty acids, etc., of a particularly noxious and evil-smelling fluid.

As in the case of diphtheria toxin the cost and labour of the production of the tetanus antigen may be reduced by this means, for the value for immunising purposes of a given toxin is that of the number of units it contains. Also, a greater certainty in immunising horses and again a saving in the cost of horses and their keep during immunisation may be confidently anticipated.

Experiments with other Cultural Filtrates.

It has already been suggested that one of these membranes might be used to form a first opinion as to the specificity or otherwise of the toxic element of any cultural fluid.

A toxic filtrate from a culture of the malignant oedema bacillus (Besson) grown in these laboratories by Mr Buxton was found to give an ultrafiltrate of undiminished toxicity. This observation indicated the probability that the toxic substance was not of the nature of a specific antigen. The filtrate in question was further investigated by Barger and Dale, who found that its essential toxicity was due to the presence of a high concentration of ammonia[1].

On the other hand, the toxic constituent of a broth culture of an organism producing a fatal epidemic in the squirrel was found to be retained by one of these bags. The suspicion that this would be a specific antigen was confirmed by Mr Buxton, who is investigating the outbreak, for he was able, using the toxin in its concentrated and purified state, to produce a corresponding antitoxin in the goat. Pressure dialysis applied to tuberculin gave in the two cases tried a complete removal of the glycerol without detectable loss of antigen. Mr Buxton, to whom I am again indebted for animal tests, employed Koch's method, using sensitised guinea pigs. For ophthalmic tests where the presence of glycerol is not permissible, or for low potency tuberculins where further concentration by evaporation is impossible, the method should prove to be of considerable utility in view of the waste and inconvenience of the alcohol precipitation method at present practised.

Mallein has also been concentrated and freed from glycerol in the same way.

[1] Private communication by Drs Barger and Dale.

Filtration of pituitary active principle, etc.

Although they are not antigens, the behaviour of secretin and the active principle of pituitary extract when filtered under pressure through these bags is of considerable interest. A simple pituitary extract gave a filtrate which, compared with the original solution and the residue, showed that although much of the active principle had passed through it had not done so at the same rate as the water, salts, etc. A similar result was obtained with the co-enzyme of zymase, in an experiment in conjunction with Prof. Harden, to whom I am indebted for the material and the tests.

Dr P. P. Laidlaw has communicated to me privately that one of his secretin preparations, filtered through a standard bag into saturated picric acid, gave at once a crystalline precipitate of marked activity.

I wish to express my indebtedness to the assistance of Mr Ralph Defries during the course of this work.

Summary.

Experiments are described in which the permeability of collodion-water membranes of a special type to various antigens was investigated. Owing to the fact that none of the antigens tried was found to pass through these membranes their use is suggested for the purpose of forming a first opinion as to the specificity or otherwise of the toxic element of any cultural fluid. The recognition and subsequent concentration of the antigen of a fatal epidemic in the squirrel was effected by this means, and an investigation of the nature of the toxicity of filtrates of malignant oedema cultures led to its complete elucidation.

Mallein and tuberculin were freed from glycerol and a considerable quantity of nitrogenous material in this way.

A process for the concentration and purification of diphtheria toxin which at a yield of 70–80 per cent. diminishes the nitrogen content about 50 times has been fully worked out. It is performed by dialysis under pressure, followed by acidification, centrifugalisation, and re-solution of the small precipitate obtained in a trace of alkali.

If a continual supply of toxin having L_+ dose 0·20 could be relied upon, this artificial conversion of low value toxin to that of high titre, would remain more of academic than practical interest. But, the majority of batches do not reach this high level and hence this concentration

process is of considerable importance. One thing is certain and that is that the periodic recurrence of "bad" toxin to which every laboratory is subjected need now no longer be feared.

By ultrafiltration followed by pressure dialysis a concentration of tetanus toxin may be effected which should be of considerable practical value, for the disintegration products of the broth materials which accompany the antigen are particularly noxious in this case.

These membranes are impermeable to enzymes, but allow to pass secretin, the pituitary active principle, the co-enzyme of zymase, and the toxic constituent of Witte peptone.

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