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

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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 anti-toxin 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.	Area exposed 1960 sq. cm. Time 24 hours.
Filtrate 8 cc., of which 1 cc. has no effect on the guinea pig. Pressure 6" water.	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 ammonia¹.

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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TABLE VIII. *Totally Depancreatized Dogs I, III, IV, V, VII and VIII.*

Date	Diet (g. per diem)	Carbo- hydrate	Fat	Urine (g. per diem)										Faeces			% of Total Nitrogen					Temp.	Pulse rate	Resp. rate	Weight	Notes																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
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