DIPHTHERIA PROPHYLACTICS*

THEIR PREPARATION AND ASSAY IN THE LABORATORY

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The real fight against diphtheria began in 1884 when Loeffler[1] isolated *Corynebacterium diphtheriae* in artificial culture. This made possible the preparation of toxin by Roux and Yersin[2] in 1888, and of antitoxin in small animals by von Behring and Kitasato[3] in 1890 and in horses by Roux and Martin[4] in 1894. Ehrlich[5] in 1897 introduced methods of standardizing toxin and antitoxin by *in vitro* methods (L+ and Lo), coined the name 'toxoid' and studied this non-toxic derivative of toxin in detail. Ramon[6] in 1922 showed that toxin and antitoxin may be titrated accurately and quickly by an *in vitro* flocculation test (Lf). Schick[7] introduced the test named after him in 1913.

In 1913, Von Behring[8] used toxin-antitoxin mixtures (TAM) for the immunization of man and in 1914 Park and Zingher[9] in New York City carried out an extensive immunization programme with this prophylactic.

Glenny and Südmersen[10] and Glenny and Hopkins[11] (1923) in Britain and Ramon[12] (1923) in France prepared formol-toxoid (FT) and in 1926 Glenny and his colleagues[13] introduced the first 'particulate' prophylactic, alum-precipitated toxoid (APT). Holt[14] in 1946 prepared another particulate antigen, PTAP—purified toxoid adsorbed on aluminium phosphate ($AlPO_4$) although, before this time, less thoroughly purified FT adsorbed on aluminium hydroxide had been used, particularly in Denmark.

Hartley[15] (1925) introduced toxin-antitoxin floccules and Glenny and Pope[16] (1927) prepared toxoid-antitoxin floccules (TAF). In 1951 Mason[17] introduced ADF—TAF dissolved in alkali and adsorbed on $AlPO_4$.

The first antitoxins in general use were the 'natural' sera of hyperimmunized horses. Later, some degree of purification and concentration was obtained by salting out the antitoxin-containing globulins with ammonium or sodium sulphate, and in recent years a marked purification and concentration has been achieved by 'refining' the antitoxic serum with pepsin (Parfentjev,[18] 1936; Pope,[19] 1938-1939; Hansen,[20] 1941).

A bibliography of all the articles leading up to modern methods of preparing prophylactics would, alone, run to many pages. Those given have an important bearing on the discussion of methods that follows.

PREPARATION OF TOXIN

Strain of C. diphtheriae. There can be few production laboratories that do not use the classical Park-Williams 8

* Presented at the South African Medical Congress, Durban, September 1957.

(PW8) strain, or a variant of it, for toxin production, because a 'wild' highly toxigenic strain is seldom encountered. In a recent investigation of 193 such strains isolated from patients in the Fever Hospital, Johannesburg, none was found to approach PW8 as a toxin producer and an attempt to 'coach' a few likely ones to produce potent toxin was unsuccessful. In these laboratories, the 'Toronto' variant of PW8 is used.

Culture Medium. The composition varies from laboratory to laboratory but, as a rule, it is an enzymatic digest of ox flesh or casein or an acid hydrolysate of casein, supplemented with amino acids, a source of carbon such as sodium acetate or lactate, salts, and maltose. The iron content is of prime importance for good toxin production and unless the medium is of a purely synthetic origin, it is nearly always necessary to carry out deferration so that the medium contains less than 0·2 μg. iron/ml. The medium used in these laboratories is a slight modification of that devised by Pope and Linggood[21] (1939)—a pancreatic digest of ox flesh, to which is added sodium acetate, sodium lactate, yeast extract and maltose.

Culture Vessels. C. diphtheriae is a strict aerobe and does not produce potent toxin unless the oxygen supply is adequate. For this reason, medium is placed in flat-bottomed flasks to form a layer not more than 2 cm. deep.

Incubation. 'Still' cultures are incubated at 35°C (not 37°C) for 6 days. A thin, translucent pellicle covers the surface of the medium in 24-36 hours, thickens considerably in 48 hours, and later forms 'curtains'. By the 6th day, a copious precipitate of deposited bacteria is present and the medium has a sherry colour due to the formation of porphyrins. Linggood *et al.*[22] (1954) have introduced an ingenious modification whereby the potency of toxin may be increased 3-4 times and the period of incubation reduced to 2 days. 'Double-strength' broth containing 4-8 times the usual amount of maltose is dispensed, not in shallow layers, but in 14 litre quantities in containers containing a propeller and an air inlet. Sterile air is introduced, is sucked down the vortex and is forced into the medium by the propeller in the form of minute bubbles. A quick, luxuriant growth, with high toxin production is the result. The method has been adopted in these laboratories.

Harvesting and Sterilization of Toxin. The cultures in the flasks are bulked, clarified through cloth coated with infusorial earth, and sterilized by candle or pad filtration.

THE TITRATION OF TOXIN AND TOXOID

Minimal Lethal Dose of a Toxin (m.l.d.)

This is the smallest amount that, injected subcutaneously, will kill a 250 g. guinea-pig within 5 days. This test measures the *toxicity* of a toxic filtrate but not its power to unite with antitoxin, or its antigenicity, i.e. its power to produce immunity.

Under the heading, 'Formol-toxoid', the preparation of 'artificial' toxoid with formalin is discussed, but what may be called 'natural toxoid', i.e. a non-toxic derivative of toxin, is present even in freshly prepared toxic filtrates and it increases in amount as the material becomes older. Thus, the m.l.d. will increase with the passage of time.

A toxin with an m.l.d. of $x/100$ ml. will most probably be, either as toxin or toxoid, a better antigen than one with an m.l.d. of x ml., but it does not follow that one with an m.l.d. of $x/2$ ml. will be twice as good an antigen as one with an m.l.d. of x ml.

The toxic filtrate with an m.l.d. of x ml. *may* contain much more natural toxoid than that with an m.l.d. of $x/2$ ml.; if so it will combine with more antitoxin and thus, other things being equal, has a better chance of being a better antigen (see under 'L+', 'Lr', 'Lf' and 'Laboratory Assay of Prophylactics').

In the preparation of prophylactics, there is little need to find the m.l.d. of a toxin because it furnishes very little useful information.

Minimal (Skin) Reacting Dose of a Toxin (m.r.d.)

This is the smallest amount of toxin that will cause a reaction within 48 hours when injected intracutaneously into a guinea-pig or rabbit. It is a much smaller amount than the m.l.d. and will, therefore, detect less antitoxin but, as for the m.l.d., there is no need, in routine toxin production, to carry out the test (see 'Lr' and 'Schick toxin').

L+ and Lo (L=limit; +=death sign; o=null sign)

The symbol L+ represents the smallest amount of toxin which, mixed with 1 unit of antitoxin and injected subcutaneously, will kill a 250 g. guinea-pig within 5 days.

The Lo is the largest amount of toxin which, mixed with 1 unit of antitoxin and injected subcutaneously, will not cause a local oedema in a guinea-pig.

Until the introduction of the flocculation test, the estimation of the L+ was the only means of finding the antitoxin-binding power of a toxic filtrate, because it also measures the toxoid content as toxoid is bound by, or unites with, antitoxin in the same way as toxin. The lower the L+ of a toxin, the higher is its antitoxin-binding value; for example, if the L+ of toxin A is 0·01 ml. and of toxin B 0·04 ml., then 1·0 ml. of A is bound by or is equivalent to 100 units of antitoxin whereas 1·0 ml. of B is equivalent to 25 units, a 4-times difference in favour of A. Experience has shown that, other things being equal, the higher the antitoxin-binding power of a toxin, the better will be its antigenicity and that of the FT prepared from it; but this value is obtained nowadays more quickly and accurately by the flocculation test.

Lr of a Toxin (r=reaction)

This is the smallest amount of toxin which, mixed with 1 unit of antitoxin and injected intracutaneously into a guinea-pig or rabbit, will cause a reaction within 48 hours. This test has the advantage over the L+ that 10-16 titrations may be carried out in one guinea-pig and 40-60 in one rabbit. But, as with the L+, there is little need, as a routine method, to find the Lr of a toxin, because the flocculation test has replaced it. However, the Lr/1000 dose of Schick toxin must be found, and the intracutaneous test is used, as a routine, to titrate antitoxin for issue (see below) and to assay sera the antitoxin content of which is too small to be found by flocculation.

Lf of a Toxin or Toxoid (f=flocculation)

This is the amount of toxin or toxoid that is equivalent to 1 unit of antitoxin as determined by the flocculation test. By experience one knows that a routinely produced toxin will have an Lf of between 60 and 100, i.e., 1 ml. will be equivalent to between 60 and 100 units of antitoxin. A unit

of antitoxin has been laid down by an international body, and standard antitoxin containing a stated number of units per ml. is issued to laboratories on request, every 6 months. With such a standard or with a laboratory standard checked against it, the Lf, L+ or Lr of a toxin may be established.

The test is carried out in the following way: Into a series of small tubes, 50, 55, 60 . . . 110 units of antitoxin and 1 ml. of toxin or toxoid under test are pipetted. The contents are mixed and the tubes placed in a water bath at 42°C. They are inspected frequently and carefully. After a period varying, according to the antigen and/or the antitoxin, from a few seconds to an hour or two, an opalescence and later floccules will be noted in one tube, the *initial* tube; still later, flocculation will take place in the tubes to the right and left of this tube. If the initial tube contains 80 units of antitoxin, the Lf of the toxin is 80, i.e. 1 ml. of toxin is neutralized by, or is equivalent to, 80 units of antitoxin. In the supernatant of this flocculated mixture, no free toxin or antitoxin (or only a trace of one or the other) will be found. This test is rapid and accurate and assays *toxoid* as well as toxin. Further, if toxin or toxoid of known Lf value is used, antitoxin may be evaluated equally quickly. However, for reasons that will be discussed later, the flocculation test must not be used as a measure of the antigenicity of a prophylactic or of the curative value of an antitoxin.

PREPARATION OF PROPHYLACTICS

Formol-toxoid (FT)

This is the basis of all prophylactics in use today. To toxin, enough formalin (40% formaldehyde) is added to combine with the free amino acids plus an additional amount found by experience; in these laboratories, the total amount added varies between 0·7 ml. and 0·9 ml. per 100 ml. toxin. The pH is adjusted to 7·2, phosphate buffer to maintain this pH is added, and the bottles are incubated at 37°C for 30 days. On 3 occasions at weekly intervals the pH is checked and brought to 7·2 if necessary. A high pH causes considerable destruction of toxin as opposed to conversion to toxoid. At the end of the 'toxoiding' process, a sample is tested got non-toxicity (5 ml. injected subcutaneously must cause no more than a transitory swelling in a guinea-pig) and for Lf value. As a rule, this falls slightly owing to destruction of antigen.

Until the introduction of APT and later of PTAP, FT was the only prophylactic used; it is still used, usually in a purified form, frequently combined with *Haemophilus pertussis*. It produces a satisfactory but not a high-grade immunity after 3 injections of 50 Lf at monthly intervals. Like most diphtheria prophylactics it is well tolerated by babies but is liable to cause reactions in some older children and adolescents.

Alum-precipitated Toxoid (*APT*)

Some degree of decolorization (removal of porphyrins) and purification of FT is usually carried out, e.g. by treatment with charcoal or Decolorite (an ion-exchange resin). The optimum amount of potash-alum to be added is ascertained, i.e. that amount that will precipitate the largest amount of antigen with the smallest nitrogen content. This amount, usually between 1% and 2%, is added to the bulk. The precipitate that forms is washed 4 times in saline and finally suspended in saline so that 0·5 ml. of the prophylactic contains 25 Lf found by flocculation after dissolving the precipitate in 2% sodium-citrate solution.

APT deservedly enjoyed a world-wide use and is only now being superseded by PTAP. It produces a solid basic immunity after 2 injections, each of 25 Lf, separated by an interval of 4-8 weeks. Its liability to cause reactions is similar to that of FT.

Purified Toxoid Adsorbed on Aluminimum Phosphate (*PTAP*)

Methods of purification vary from laboratory to laboratory and include removal of pigment on magnesia gel, charcoal or ion-exchange resin; concentration by ultra-filtration or fractional precipitation by ammonium sulphate followed by dialysis; further fractional precipitation by ammonium sulphate; and adsorption on, and elution from, cadmium chloride followed by dialysis. It is relatively easy to increase the purity of a crude toxoid of 150-200 Lf/mg. protein nitrogen (PN) to 1800-2000 Lf/mg. PN, but much more difficult and somewhat wasteful to increase this to 2500-3000 Lf/mg. PN.

In these laboratories, most of the colour and a considerable amount of nitrogenous impurity is removed on charcoal and on Decolorite; 40 g. of ammonium sulphate—$(NH_4)_2SO_4$—is added to each 100 ml. of decolorized toxoid, the precipitate is collected and washed with 40% $(NH_4)_2SO_4$ and then dialysed in a Cellophane bag against very cold running water for 5 days. The contents of the bag are clarified and the toxoid is precipitated with 0·75% cadmium chloride. After being washed in 0·5% cadmium chloride the precipitate is dissolved in 5% sodium phosphate (Na_2HPO_4). The solution is dialysed for 4 days against cold running water, clarified, made isotonic with NaCl, preserved with 0·01% Merthiolate, and finally sterilized by candle filtration. The purity varies between 1,800 and 2,000 Lf/mg. PN.

Purified toxoid is, Lf for Lf, no better and probably worse than crude FT as an antigen, but when adsorbed on $AlPO_4$ it is much superior. It is added as a concentrate (4,000-6,000 Lf/ml.) to an $AlPO_4$ suspension at pH 5·7 so that the PTAP for issue will contain 25 Lf and 2·5 mg. $AlPO_4$ per 0·5 ml. A period of 3 and preferably of 6 months' storage at about 20°C is necessary for a firm adsorption of the toxoid to the $AlPO_4$ to take place.

PTAP is a very good antigen, producing a solid basic immunity after 2 injections of 25 Lf at 4-8 weeks' interval. It causes fewer reactions than FT or APT.

Dissolved Toxoid-antitoxin Floccules (*DF*) *and Adsorbed Dissolved Floccules* (*ADF*)

On *a priori* grounds it could be assumed that the diphtheria toxoid moiety of floccules would be purer than a toxoid purified by physico-chemical means. If toxoid alone flocculated from a crude FT, it would be absolutely pure but, unfortunately, other constituents 'come down' in the floccules. But by gel diffusion tests, it has been shown that DF is purer than purified prophylactics regularly issued. By a gel diffusion technique, Pope *et al.*[23] (1951) demonstrated 14 antigens in a toxoid containing 2,170 Lf/mg. N whereas, by a similar method, no more than 4 have been found in DF.

DF is prepared by flocculating FT with refined antitoxin at the 'initial tube' level. The floccules are spun out and washed 4 times with saline. They are then dissolved in N/20 NaOH and the alkali allowed to act for 1 hour at room temperature. This treatment destroys all but a minute

trace of the antitoxin. The alkali is neutralized with HCl, and NaCl to isotonicity and 0·01% Merthiolate are added; the concentrated DF solution is sterilized by candle filtration. Thereafter, the adsorption on to $AlPO_4$ to produce ADF is the same as that noted under 'PTAP'.

The immunity produced by 25 Lf of ADF followed in 6-8 weeks' time by another 25 Lf of ADF or, preferably, of DF is of the same order as that obtained with PTAP. Possibly it produces fewer reactions than PTAP in older children and adolescents.

Combined Prophylactics

PTAP may be mixed with a suspension of *H. pertussis* or with *H. pertussis* and purified tetanus toxoid to form combined prophylactics for the immunization of man against diphtheria and whooping cough or these two diseases and tetanus simultaneously.

Schick Toxin

A Schick dose of toxin is that amount which causes a reaction when it is mixed with 1/1,250 unit of antitoxin and injected intracutaneously into a guinea-pig but does not cause a reaction when mixed with 1/750 unit; further, 1/25th of a Schick dose, without antitoxin, shall cause a reaction and 1/50th shall cause no reaction. In other words, a Schick dose of toxin is equivalent to 1/1,000 unit of antitoxin and contains 25 m.r.d.

In practice, toluene-preserved samples of toxin are set aside in the cold room and allowed to 'lager' for a year or more until toxoiding and destructive processes have become stabilized. They are then titrated and one containing 25 m.r.d. per Lr/1000 dose is chosen.

The Schick test is much more of a qualitative than a quantitative method of measuring immunity. People with 0·004 unit or more of antitoxin per ml. of serum will not react whereas those with less will react. But as at least 0·04 unit per ml. of serum is necessary to prevent an attack of diphtheria, nothing definite can be said of the 'real' immune state of a Schick-negative person. The test shows that he has some degree of immunity but not how much.

It is essential to inject the amount of Schick toxin prescribed by the manufacturers and not an amount that will produce a bleb in the skin, because this may be much less than the correct dose. A control injection of heated toxin must be given to pick out pseudo-reactors.

The test has its main application in mass survey when some idea of the immune state of a community is desired.

LABORATORY ASSAY OF PROPHYLACTICS

If every laboratory in the world used the same strain of *C. diphtheriae*, if this never varied in its metabolism, if the medium used was completely reproducible, and if all other conditions—temperature, period of incubation, method of preparing FT, purification etc.—were the same, the necessity for assaying a prophylactic for antigenicity would hardly arise. But this ideal state of affairs does not prevail.

In these laboratories, toxin is prepared weekly and, whilst the analysis of the culture medium varies only slightly from batch to batch, it does vary. Again, the Lf of each toxic filtrate is not the same, nor is the chemical composition of every FT. Although each purified toxoid contains between 1,800 and 2,000 Lf/mg. PN and the results of assays of different batches of PTAP for antigenicity are not, statistically, significantly different, it must be realized that the Biological Control Laboratory is not legislating for one laboratory only but for any that may issue a diphtheria prophylactic.

The test at present in force is a 'minimum requirements test', i.e. one that demands a specified minimum immunity in a specified number of guinea-pigs immunized with one or more specified doses of prophylactic. In the Institute, 10 or more guinea-pigs receive, subcutaneously, 1 Lf of PTAP or ADF in 0·5 ml. of $AlPO_4$ suspension containing 2·5 mg. of $AlPO_4$. This amount is again injected 28 days later and, 14 days after this, blood is removed from each animal. The antitoxin content of each serum is assayed at the Lr/1000 level in guinea-pigs and the geometric mean of the 10 or more values obtained is worked out. No prophylactic would be issued unless this value was at least 2 units per ml. of serum but, in practice, it is always between 5 and 10.

This is a satisfactory test—up to a point. It shows, for example, that a particular prophylactic is a satisfactory antigen when assayed in Johannesburg, in summer, in in-bred guinea-pigs fed on a good diet and housed in good conditions. But would the same result have been got if it had been tested in winter, in 'mongrel' guinea-pigs, badly fed and housed in another part of the world? In other words, it is possible that a prophylactic, passed for issue in South Africa, would be rejected in another country.

Little or no difficulty is encountered in titrating an antitoxin, because it can be compared under identical conditions with an international standard antitoxin of given value. International bodies are working on the preparation of a standard antigen with which an unknown prophylactic could be compared. When such a standard is available, the country, season, animal and food will play no part because whatever prevails for the prophylactic will prevail for the standard.

However, work being carried out in these laboratories indicates strongly that, where particulate prophylactics are concerned, many difficulties will have to be overcome before a generally acceptable method is devised. For example, will the test demand 1 or 2 injections, how many Lf will be used per injection, will the primary stimulus be larger or smaller than the secondary, and will the test involve the immunization of groups of guinea-pigs, the first with xLf the second with $x/2$ Lf and the third with $x/4$ Lf?

If a 1-dose method is adopted, it might lead to the rejection of a prophylactic that would pass easily by a 2-dose test. If 2 doses are stipulated, other difficulties arise. If the Lf dose is very small, it is possible that some eminently satisfactory prophylactics would fail and, if large, that some weak antigens would pass. Although some of the work going on is, possibly, of more academic than practical value, it is right that it should continue until a fully satisfactory test is worked out. The laboratory worker wants to prepare, and the physician wants to use, the best product available.

PREPARATION OF ANTITOXIN

The horse is the most important single factor involved in antitoxin production because, as producers, some animals are good and others bad. Naturally, the most suitable toxin, size of dose and spacing of injections, as shown by experience, are used, but a 'bad' horse cannot be turned into a 'good' one

by, for instance, injecting double the usual amount of antigen into it.

A toxin made in a pancreatic digest of ox flesh (see under 'Preparation of Toxin') is unsuitable because, in the large doses required for hyperimmunization, it upsets the animal and causes large swellings. Martin's broth, a peptic digest of pig's stomach and beef, is suitable because toxin made in it is reasonably well tolerated and it produces the desired result in most horses, viz. a high-titre antitoxin.

Each horse that enters a serum-production stud must pass the mallein and tuberculin tests (the last case of glanders was seen in South Africa in 1923 and tuberculosis is rare in the horse). Each horse's serum is then titrated for the presence of 'normal' diphtheria antitoxin by the intracutaneous method and only those horses with at least 0·1 unit per ml. are allocated to the diphtheria section of the stud. Among the last 100 horses entering the stables 14 were found to contain 1 or more units per ml. of serum, 41 between 0·1 and 1 unit and 45 less than 0·1 unit. The presence of 'normal' antitoxin has no connection with recovery from clinical diphtheria because the horse is not susceptible but is due to small, repeated, stimulations by the toxin of *C. diphtheriae* which may be found in abrasions, wounds and mucosae.

The 'natural' immunity of a horse provides a flying start to hyperimmunization. There is no need to 'prepare' the horse by 2 or more injections of APT and to wait for 6-12 months before starting a course as must be done when horses have to be hyperimmunized against tetanus toxin because 'normal' tetanus antitoxin is found only rarely in horses, if at all.

Some laboratories start immunizing with FT and use unmodified toxin only in the later stages, but in this Institute toxin is used from the start. The first few doses are small and are given daily until an amount of 5 ml. is reached. Thereafter, increasingly larger amounts are injected subcutaneously, thrice and later twice weekly until the dose is 300 or even 400 ml. According to the antitoxin response and physical condition of the animal, this primary course lasts 4-6 weeks. When a test shows that the serum antitoxin titre is 1,000 or more units/ml., 20 litres of blood are removed, (2 × 10 litres with a 4-day interval between bleedings) from the jugular vein into citrate solution. The horse is rested for 4-5 weeks, undergoes a rapid re-immunization lasting 10-14 days, is bled, is again rested and so on until its serum-antitoxin titre falls to 800 units/ml. or less. This happens at any time between the 5th and 15th course.

Concentration and Purification of Antitoxic Plasma:

To plasma containing 0·3% cresol and diluted with 2 parts of water, enough pepsin is added to make the concentration 0·25% and the pH is brought to 3·2. After the mixture has stood for 40 minutes at room temperature, ammonium sulphate, to 14%(w/v), is added and the pH is adjusted to 4·3. The precipitate that forms after heating at 55°C for 1½ hours is discarded and ammonium sulphate to a total of 31% (w/v) is added to the filtrate at pH 7·4. The precipitate is collected on filter pads, is pressed, and is dialysed in Cellophane bags for 6 days against cold running water.

The concentrated antitoxin globulin solution, preserved with 0·3% cresol, is clarified and diluted with 0·3% cresol-saline to contain about 4,800 units per ml. as ascertained by the flocculation test. A preliminary *in vivo* titration is then carried out intracutaneously and sufficient cresol-saline is added to bring the value to 4,500 units/ml. The antitoxin solution is then sterilized by pad filtration and another intracutaneous test carried out to confirm that it contains at least 4,400 units per ml.

Sterility tests in culture media and safety tests (5 ml. subcutaneously in the guinea-pig and 1·0 ml. in the mouse) must be passed before the refined antitoxin is put into ampoules. The unitage of a few of these is checked *in vitro* and *in vivo*. Sterility and safety tests of the contents of ampoules taken at the start, the middle and the end of the filling process must be passed. The antitoxin is then ready to be issued.

Antitoxins may be 'avid' or 'non-avid', i.e. they vary in their ability to form a firm union with toxin. The unitage assigned to a non-avid antitoxin by the flocculation test will be higher than that obtained by an *in vivo* titration (L+ or Lr type of test) because when the toxin-antitoxin mixtures are diluted in body fluids after injection into a guinea-pig, they tend to dissociate. Thus, more antitoxin is required to neutralize the test dose of toxin in the animal than is needed for the same amount of toxin in the flocculation test. Biological control laboratories rightly demand the animal test as the method of titration.

In these laboratories, the *in vitro/in vivo* ratio of every diphtheria antitoxin issued is about 1, because each batch is made from the pooled sera of at least 20 horses; the effect of the possible presence of one or two non-avid sera is thus minimized.

The increase in purity obtained by salting-out methods (ammonium or sodium sulphate) is only about 50% whereas it is about 300% by the pepsin-digestion process (units of antitoxin per gram of protein). Pepsin splits the antitoxin-containing globulin molecule into two, and renders that portion of it that is poor in antibody heat-coagulable in the presence of ammonium sulphate. This part is discarded in the first precipitate. Refined antitoxin is much less liable to cause serum reactions, either immediate or delayed, than natural serum or sulphate precipitates.

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