XCIX. THE PREPARATION AND SOME PROPERTIES OF PURIFIED DIPHTHERIA TOXOID.

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The desirability of purifying toxins and antigens in general need not be emphasised. One of the chief difficulties experienced in the employment of the Schick test for susceptibility to diphtheria is the presence in the toxic filtrates of some substance which produces the "pseudo" reaction and hinders the correct interpretation of the specific effect due to the toxin. A similar reacting substance appears to exist in the filtrates of the streptococcus which have recently been used in the Dick test for susceptibility to scarlet fever [Dick and Dick, 1924]. In both cases the substance causing the "pseudo" reaction is definitely more heat-stable than the specific substance, but beyond this fact nothing is known of its constitution or properties. Further, many culture filtrates to a greater or less degree contain substances which tend to interfere with the general condition of the animal being immunised [Watson and Langstaff, 1926]. These substances may cause temperatures and other non-specific reactions which preclude the injection without injury of the increasingly large doses of toxin necessary for the successful production of antitoxin. The presence of similar non-specific substances has recently been suspected in the culture filtrates of *B. Welchii* and *B. coli* by Dalling and Mason [1926]. The production of immunity to lamb dysentery was interfered with and in some cases lameness and even death caused by some component of the culture filtrates which formed the basis of their prophylactic mixtures. Similar difficulties exist in the clinical aspects of the problem. The active immunisation of children and adults against diphtheria and scarlet fever is attended in a small percentage of instances by painful reactions which seem to be due to some non-specific constituents of the toxin-antitoxin mixtures at present in use and not to the active principle itself. The clinical use of culture filtrates treated with formaldehyde has been explored by Park and his colleagues [1924] in America. They found that such toxoid preparations were valuable antigens [see also Glenny and Hopkins, 1923] but concluded that the toxoid preparations which had no toxicity in guinea-pigs could not be properly used in human beings in doses larger than 0·5 cc. because of the marked "pseudo" reaction. With some of the later toxoid preparations, these workers find that the difficulties connected with the local reactions have been

partially overcome. Larson and Eder [1926] also support the use of simplified immunising preparations and claim that toxic culture filtrates which have been detoxified by sodium ricinoleate are fully equal, and in many respects superior, to toxin-antitoxin mixtures as immunising agents.

There would seem to be two urgent problems awaiting solution in this field of immuno-chemical research. The first problem is to convince ourselves that under no circumstances are the toxin-toxoid reactions reversible and to determine the relative values of such detoxifying agents as formaldehyde and the salts of ricinoleic acid, etc. The work of Glenny and his colleagues in England, Ramon in France, and Park and his co-workers in America have failed up to the present to expose any sign of such a reversal in the case of culture filtrates detoxified with formaldehyde but information on the nature of the action of the formaldehyde is lacking. Our knowledge of the action of salts of ricinoleic acid on toxin is confined to the work of Larson and his co-workers and no extended use of their toxoids as immunising agents has yet been possible. The second problem awaiting solution is to purify these toxoid antigens. The active principle of culture filtrates is probably confined to a very small fraction of the total material and as shown later, it is possibly associated with less than 1 % of the nitrogenous material in the culture filtrates. If this is so, the purification of the antigens should do much to eliminate the non-specific reactions which as Park and his colleagues state preclude the proper use of atoxic preparations for the immunisation of human beings. Further, investigation of the constitution of antigens and the chemical processes of immunity may be facilitated if comparatively pure antigens are substituted for the highly impure culture filtrates which at present form the basis of our attack on the various problems.

Research in this field has been facilitated during the past few years by the work of Ramon [1922], whose flocculation test for assaying the strength of diphtheria toxin and antitoxin has received almost universal recognition. The present work is essentially a development of that of Watson and Wallace [1924, 1, 2] on the purification of culture filtrates. Some of the properties of purified toxin were described but at the time the only well-established methods of testing the various fractions were by the use of animals, and progress was necessarily slow. This work has recently been confirmed by Moloney and Weld in Canada [1925]. The present paper consists of a brief survey of the method of purification of culture filtrates containing toxins partially or completely converted into toxoids by formalin together with a discussion of some of the properties of the purified substance. The technique of Ramon was employed for testing the value of the fractions and has thrown light on many of the facts which were described in the previous paper [Watson and Wallace, 1924, 2] and for which at the time no explanation was forthcoming.

Terminology.

A survey of the terminology at present in use in the field of immunological research has recently been made by Glenny, Pope and Waddington [1925]. The term *purified toxin* is used in the present paper to denote the active fraction obtained from culture filtrates of the diphtheria bacillus by precipitation at low temperature with glacial acetic acid and rapid separation of the precipitate, which is then dissolved in cooled dilute caustic soda to p_H 8·0 [Watson and Wallace, 1924, 2]. *Purified toxoid* is employed to denote the active fraction obtained from diphtheria toxoid by precipitation at 35° or less with glacial acetic acid and separation of the precipitate which is then dissolved in dilute caustic soda to p_H 8·0. Many of the purified toxoid solutions used during the course of the work were not completely atoxic, the original toxins having been only partially converted into toxoids before concentration. The terms *ultra-purified toxin and toxoid* are employed to denote purified toxin or toxoid which has been subjected to further purification by means of acetic acid precipitation, or alcohol precipitation [Moloney and Weld, 1925] or by dialysis against water for periods of 48–72 hours.

Methods.

The media used for culture filtrates were prepared by different variations of enzyme digests of horse muscle [Watson and Langstaff, 1926] or by the solution of commercial peptones in muscle extracts. The media, sterilised by filtration through a Seitz filter press followed by a short steaming, were inoculated with Park William No. 8 strain of *C. diphtheriae* and incubated for 10 days in the case of digest medium and 7 days in the case of peptone medium. To obtain diphtheria toxin, the filtrates from each culture were then tested and graded accordingly. Each group of cultures was filtered through a Berkefeld candle either without preservative or else with 0·5 % added phenol or toluene. To prepare diphtheria toxoid a similar technique was adopted but the cultures were treated directly with 0·5 % neutralised formalin. The filtrates were either concentrated at this stage or else were incubated for varying periods (sometimes with the addition of extra formaldehyde) to complete the toxin-toxoid change. A preliminary assay of a group of bulked and filtered toxoids was carried out. Total, Van Slyke, and precipitable nitrogen [Watson and Wallace, 1924, 1] and total solids content were estimated. The Lf value[1] was also determined in each case. A similar series of data was accumulated for the purified solutions and from these observations some idea could be obtained as to the degree of purity that had been reached relative to the original unpurified toxoid.

[1] The Lf dose is that amount of "toxin" which is equivalent to one unit of antitoxin by the flocculation test [Glenny and Okell, 1924].

THE ACTION OF ACIDS AND ALKALIS ON FORMALISED CULTURE FILTRATES OF *C. DIPHTHERIAE*.

The action of a strong acid, *e.g.* hydrochloric acid and a strong base, *e.g.* sodium hydroxide on a typical formalised culture filtrate is shown in Fig. 1 (Curve A).

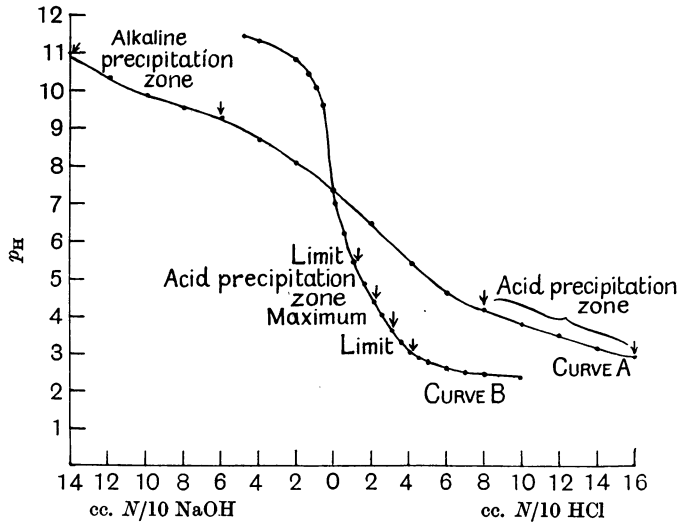

Fig. 1. Buffer curves of diphtheria toxoid and purified diphtheria toxoid.
Curve A. Diphtheria toxoid. Curve B. Purified diphtheria toxoid.

Varying amounts of decinormal acid and alkali were added to the filtrate and the p_H of the various samples estimated electrometrically[1].

By plotting the amounts of acid or alkali added against the corresponding p_H of the solution we obtain the "buffer" curve of the filtrate. The curve obtained in this case is typical for culture filtrates prepared from medium made by the prolonged tryptic digestion of horse muscle [Watson and Langstaff, 1926]. There are two well-marked precipitation zones, (*a*) an alkaline zone extending from p_H 9·25 to 11·0, and (*b*) an acid zone extending from p_H 2·9 to 4·2. The alkaline precipitate consists mainly of phosphates while the acid precipitate is composed of material of high molecular weight which experimental work has shown contains a proportion of the active principle of the filtrates. The action of other acids is similar to that of hydrochloric acid but the amounts of acid required to reach the precipitation zone depend, of course, on the degree of dissociation of the acid. Acetic acid is the most useful from many points of view. To obtain the most active fractions each batch of filtrates should be subjected to a separate study and the acid precipitation zone carefully explored. The technical details of such studies and their application to the "working up" of filtrates to the maximum advantage

[1] We are indebted to our colleague Mr Pope for the electrometric determinations of p_H carried out during the course of the work.

will be dealt with in a future publication. For the present work, however, 1 % glacial acetic acid has been used as the precipitating agent and the precipitate so obtained subjected to a systematic chemical study. It is of interest here to include data on the amount of precipitate produced by the action of 1 % glacial acetic acid on various types of culture filtrate. The presence of phenol or formaldehyde plays little, if any, part in determining the amount of precipitate formed. Table I gives the results obtained. For the estimations, 300 cc. of the culture filtrate were precipitated with 1 % glacial acetic acid and the precipitate coagulated by heating in a water-bath to 100° for 10 minutes. The solution was then cooled to room temperature and filtered. The precipitate was washed with 300 cc. of distilled water, transferred to a Kjeldahl flask and the nitrogen estimated in the usual way.

Table I. *Nitrogen precipitable by* 1 % *glacial acetic acid from various types of culture filtrates.*

Type	No.	Total nitrogen mg./100 cc.	Precipitable nitrogen mg./1000 cc.	Percentage: Precipitable nitrogen	Percentage: Non-precipitable nitrogen
Peptone X + horse muscle extract	J 3543	442·4	21·7	0·5	99·50
Peptone Y + horse muscle extract	J 3558	281·4	50·8	1·82	98·18
Peptone Z + horse muscle extract	J 3541	320·0	44·5	1·4	98·60
Tryptic digest (*a*) 3·5 hrs. at 40°.					
Horse muscle (Watson and Wallace)	J 3550	439·6	42·5	0·97	99·03
	J 3554	436·8	40·9	0·94	99·06
(*b*) 2·5 hrs. at 37°. (Watson and Langstaff)	TA 20	250·5	30·27	1·21	98·79
	TA 21	245·0	11·93	0·49	99·51
(*c*) 72 hrs. at 15°. (Watson and Langstaff)	TME 99	180·6	33·6	1·86	98·14
	TME 100	277·4	11·7	0·42	99·58

In all cases the amount of nitrogenous material removed from the filtrate is very small and it would appear that the active principle, whatever its nature, must form only a small percentage of the total material of the culture filtrate.

The general technique for preparing purified toxoid.

The method is essentially that of Watson and Wallace [1924, 2] and consists of the acetic acid precipitation of the culture filtrates of *C. diphtheriae* which have been treated with formalin. If completely atoxic solutions are required the formalised filtrates are incubated until the M.R.D. [Glenny and Allen, 1921] of the filtrates is negligible. The distribution of the active principle between the precipitate and supernatant fluid varies with the type of filtrate used and also with the presence of preservatives or other substances which may affect the stability of the toxoid molecule. The details of the large scale methods for precipitating culture filtrates and the working up of "super-

natants" to the best advantage will be dealt with in a future publication but the following brief outline of the process may be included here.

Formation and separation of the precipitate. The filtrates are placed in double Winchester quart bottles, 4 litres per bottle, and 1 % glacial acetic acid is added. The precipitate produced is allowed to settle out and is then removed by centrifugation. When small quantities of filtrate are being handled, this may be done in glass pots in a large centrifuge. After the preliminary centrifugation the supernatant is carefully siphoned off and replaced by an equal quantity of 0·7 % saline or sodium acetate-acetic acid mixture adjusted to the p_H of the acid precipitation point (*ca.* 3·8). If distilled water or saline of p_H above or below this point is used appreciable losses may take place during the washing. One washing is usually sufficient, but if a more complete removal of pigment from the precipitate is required one or two more washings will effect this. If large volumes of toxoid are being employed the Sharples centrifuge offers many advantages, and the removal of the precipitate may be carried out almost quantitatively if the interior of the bowl is lined with a sheet of celluloid. One drawback with this type of centrifuge is that the final washing of the precipitate cannot be carried out as satisfactorily as with a large centrifuge having glass pots. From every other point of view, however, the Sharples machine is to be preferred for the large scale concentrations.

Removal and solution of the precipitate. The celluloid cylinder on which the precipitate collects is removed from the metal bowl of the centrifuge with forceps and the precipitate scraped into glass mortars and dissolved in physiological saline or distilled water buffered to p_H 8·0. Precautions to keep the solution under ice-cold conditions such as are necessary in the production of purified toxin would appear to be unnecessary in the preparation of purified toxoid from most culture filtrates.

Chemical studies of these purified antigens can be carried out more conveniently if no preservatives are present. Phenol especially, as will be shown later, interferes with the stability and chemical properties of the solutions.

Factors influencing the preparation of purified toxoid. It was shown in the previous communication that the amount of acid used, the temperature of precipitation and speed of solution of the precipitate, all played an important part in determining the value of purified toxins. Purified toxoid is a much more stable substance. For most toxoids the temperature of precipitation (provided it is less than 37°), and the speed with which the precipitate is brought into solution, play only a small part in determining the strength of the purified solution as judged by Lf measurements. The amount of acid added still remains an important factor. As a general rule, the smallest amount of acid consistent with the production of an appreciable precipitate seems to give the best results. This amount of acid should be found experimentally for each batch of filtrates.

A typical concentration of diphtheria toxoid. Table II shows the results of

a typical concentration. Forty-eight litres of a toxoid containing 8·5 Lf units per cc. were precipitated with 1 % glacial acetic acid in 12-litre batches. The precipitated toxoid was spun in the Sharples centrifuge and the precipitates dissolved in 1500, 750, 375 and 187·5 cc. of saline respectively, the p_H adjusted to 8·0 and the solutions filtered through a Berkefeld candle. The total nitrogen and Lf values of the unfiltered samples and the total solids content and Lf value of the filtered samples were determined.

Table II. *The preparation of purified toxoid using the Sharples centrifuge for separation of precipitate.*

	Purified toxoid			
	Lf units per 10 cc.	TF 45°* (min.)	Total N mg./100 cc.	Lf units per 100 mg. N
CT 15 A	200	105	12·5	16,000
CT 15 B	460	90	21·3	21,590
CT 15 C	1040	8	60·3	17,250
CT 15 D	1800	3	90·3	19,930
	Filtered purified toxoid			
	Lf units per 10 cc.	TF 45°* (min.)	Dry weight mg./10 cc.	Total solid per Lf unit (mg.)
CT 15 A	200	65	125·5	0·627
CT 15 B	360	40	116·0	0·322
CT 15 C	720	3	120·5	0·167
CT 15 D	1300	10–8 at R.T.	102·0	0·078

* TF 45° = flocculation time at 45°.

Several facts are seen from the table. These precipitates may apparently be dissolved to give almost any required concentration of Lf units, the volume of the purified solution being limited only by the solubility of the material. The greater the strength of the solution the more rapid is the flocculation with antitoxin. The strongest sample, for instance, flocculated in a few minutes with almost an equal volume of serum containing 200 units of antitoxin per cc. If the antigenic value of a solution depended on the strength of the toxoid and the rapidity of its flocculation with antitoxin alone (which is unlikely) these purified solutions should be ideal antigens. Apart from this aspect of the problem—which is, of course, the most important one—these solutions offer us a more satisfactory starting point for researches on the chemical constitution of antigens and the part they play in immunity processes than the culture filtrates of the bacillus. The total solids content of the most potent filtered purified toxoid of the series was only 0·078 mg. per Lf unit, and when it is remembered that a considerable percentage of this was added salt the very small proportion of a culture filtrate which is occupied by the active fraction will be realised. The active principle—whatever its nature—is adsorbed to some extent by kieselguhr as will be seen by the differences in value of the solutions before and after they have been filtered through a Berkefeld candle. This adsorption increases with the amount of precipitate in solution. Once a purified solution is produced it can be diluted to any required Lf value with saline or other purified toxoid of known Lf value.

THE ULTRA-PURIFICATION OF PURIFIED SOLUTIONS.

The active fraction precipitated from the culture filtrates can be purified still further by such methods as reprecipitation with a small amount of acetic acid, or with alcohol [Moloney and Weld, 1925], or by the dialysis of the solutions in a collodion membrane against water. The degree of purity produced by any of these methods depends on the nature of the active fraction and the type of filtrate from which it was produced.

The estimation of the degree of purity of the solutions. As a simple estimate of the degree of purity attained during the stages of the concentration process the total solids and nitrogen content of one Lf unit were calculated for each of the unpurified toxoids and the various purified solutions. The ratio of the figure obtained for the purified toxoid to that for the unpurified solution gives the purity of the solution relative to the original material. The total solids concentration figures are, of course, dependent on the nature of the solvent used for the precipitate and the amounts of acid and alkali used for the p_H adjustment. The nitrogen concentration figures provide the more true estimate of the degree of purity reached. This degree of purity varies over a wide range. The active fractions may contain from 30 to 130 times less nitrogen than the original toxoid. Some of these purification figures are collected in Table III.

Table III. *The concentration of the active principle, as shown by the dry weight and total nitrogen ratios of one Lf unit.*

	No.	Total solids mg. per cc.	Total N mg. per cc.	Lf units per cc.	"Weight" of one Lf unit mg.	Nitrogen content of one Lf unit mg.	"Weight" ratio	Nitrogen ratio
Parent toxoid	TME 99	24·75	1·806	8·5	2·91	0·212	—	—
Purified toxoid	CT 15 A*	12·5	0·125	20	0·625	0·00625	4·65	33·9
	CT 15 B*	11·6	0·213	36	0·322	0·00592	9·04	35·9
	CT 15 C*	12·05	0·603	72	0·167	0·00837	17·42	25·4
Parent toxoid	TME 100	29·00	2·774	12	2·42	0·231	—	—
Purified toxoid	C4/21	1·75	—	40	0·0437	—	55·38	—
	C4/28*	0·6	—	26	0·023	—	105·22	—
	C4/34 B*	0·75	—	14	0·054	—	44·82	—
Parent toxoid	JM 3801	27·20	2·536	5·5	4·94	0·461	—	—
Purified toxoid	CT 16 A	—	0·175	36	—	0·00486	—	94·8
	CT 16 B	—	0·434	78	—	0·00556	—	82·9
	CT 16 C	—	0·895	165	—	0·00543	—	84·9
Parent toxoid	BJ 3766	—	2·755	4·5	—	0·612	—	—
Purified toxoid	CT 17 A	—	0·218	44	—	0·00495	—	123·64

* Precipitate dissolved in distilled water.

No extended investigation has been carried out on the dialysis of purified toxoid but it may be of interest to include here data with regard to the dialysis of some purified solutions obtained from phenolised culture filtrates. The purified solutions can usually be dialysed for 48–72 hours against water in a collodion membrane without loss of active principle. Table IV shows the increase in the concentration of Lf units per mg. of nitrogen in the ultra-purified solution as the dialysis proceeds.

Table IV. *The increase of purity of two reconstructed antigens as dialysis proceeds.*

Period of dialysis (hours)	Concentration of Lf units per mg. N: Filtrates prepared from tryptic digest media. CJ 24 A	Filtrates prepared from peptone media. CJ 26 B
0	81·4	146
24	112·0	161
48	141·0	182
72	146·0	194

By these methods of purification, therefore, solutions which are over 100 times as "pure" as the original culture filtrates may be obtained. It is interesting to note that Hartley [1926] working with the washed precipitates from toxin-antitoxin mixtures has recently prepared active antigens which contained less than 0·002 of the nitrogen of the mixtures from which they were derived.

THE STABILITY AND CHEMICAL PROPERTIES OF PURIFIED TOXOID.

Except where otherwise stated, the material used in the following experimental work was prepared by precipitation of diphtheria toxoid with glacial acetic acid, centrifugation of the solutions in the large centrifuge and careful washing of the precipitate before solution in dilute caustic soda to p_H 8·0. No preservative was used for the purified solutions unless otherwise stated.

The stability of purified toxoid at various temperatures.

(*a*) *Temperatures below freezing point.* The effect of freezing purified toxoid in the presence of phenol (0·5 %), formalin (0·5 %), hydrogen peroxide (0·5 %) and without preservative is shown in Table V.

Table V. *The effect of freezing purified toxoid with and without antiseptic.* (TME 100. C 4/19.)

Solution	p_H	Preservative	Lf units per cc.: Before freezing	After freezing for: 3 hours	20 hours
Original toxoid (TME 100)	7·2	Formalin	12	12	12
Purified toxoid	8·0	—	12	12	12
,,	8·0	0·5 % phenol	12	8	3
,,	8·0	0·5 % hydrogen peroxide	12	12	12
,,	8·0	0·1 % formalin	12	12	No flocc.

No destruction was observed after freezing the original toxoid for 3 or 20 hours. The purified toxoid, whether unpreserved or preserved with formalin or hydrogen peroxide, suffered no ill effects from short or prolonged freezing. 75 % of the flocculating value was removed by freezing the purified toxoid when preserved with phenol. The deleterious effect of the presence of phenol as a preservative for culture filtrates was pointed out by Moloney and Weld

[1925] and Glenny, Pope, Waddington and Wallace [1925] demonstrated the ill effects of freezing toxin-antitoxin mixtures in the presence of phenol.

(*b*) *Temperatures* 5°–37°. The stability of purified toxin within this temperature range was dealt with in a previous paper [Watson and Wallace, 1924, 2], where it was shown that deterioration or modification proceeded rapidly at 37° and definitely but more slowly at 17° and 5°. After some time at the latter temperatures a state of comparative equilibrium was set up. The values of the various samples after different time periods were estimated by animal tests (Lr/500 skin measurements). The stability of purified toxoid as measured by Lf tests is well marked. Without preservative it may be kept for long periods of time without loss of flocculating power with antitoxin. The presence of phenol tends to definite destruction of the flocculating value on standing. If formalin is added to these purified toxoids, even in minute amounts, flocculation with antitoxin may be difficult to detect or the time of flocculation is increased. These facts are illustrated in Table VI.

Table VI. *Stability of purified toxoid with and without preservative at various temperatures.*

Lf units per cc.

Period in days	No preservative			Phenol 0·5 %			Formalin 0·1 %			Hydrogen peroxide 0·5 %		
	4°	17°	37°	4°	17°	37°	4°	17°	37°	4°	17°	37°
0	16	16	16	14	14	14	14	14	14	14	14	14
1	16	16	16	14	14	12–14	12	12	12	14	14	14
2	16	16	16	—	—	—	(Time of floccula-			—	—	—
4	16	16	16	—	—	—	tion 6 days)			—	—	—
7	—	—	—*	14	14	10				14	14	14
11	16	16	10	—	—	—				—	—	—
15	—	—	—	14	14	10				14	14	14
18	16	16	—	—	—	—				—	—	—
23	—	—	—	14	14	10				14	14	14
26	16	16	—	—	—	—				—	—	—*
30	16	16	—	14	14	8				14	14	10
44	16	16	—	14	14	8				16	16	10
65	16	16	—	14	14	—				16	16	—

* Contaminated.

Some of these purified solutions have been kept for over 2 years at cold room temperature and in some cases they still retain their high flocculating power. It would seem that the "deterioration or modification" of the purified toxins described in the previous paper may have been one of modification only. The purified toxic component of the solution apparently slowly changes into the toxoid component, the reaction being facilitated by warmth.

(*c*) *Temperatures above* 37°. The behaviour of the purified toxoid without antiseptic when heated to temperatures beyond 37° is shown in Fig. 2. About 200 cc. of purified toxoids of different initial flocculating values were placed in flasks on an electric heater and taken slowly to 100° with constant shaking in the presence of glass beads. Samples were removed at various temperatures and examined for p_H and Lf values. The p_H changes of the various samples were negligible. There are indications of slight destruction before a temperature

of 100° is reached, after which complete destruction takes place very quickly. There is also some indication that the stronger toxoid is slightly more heat-labile than the weaker—a point which has been suggested by other similar experiments.

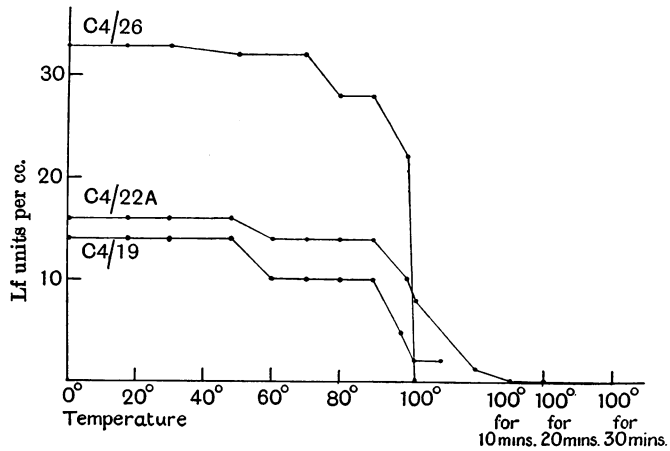

Fig. 2. The effect of heat on purified toxoids without preservatives.

The stability of purified toxoid to acids and alkalis.

Fig. 1 (Curve B) shows the buffer curve of a typical purified toxoid. The solutions were treated with $N/50$ acid and alkali. There is no alkaline precipitation zone such as exists in the case of the unpurified filtrates. The small buffer content of the solution is illustrated by the steepness of the curve. Precipitation begins at p_H 5·5 and the precipitate is redissolved at p_H 2·8. Maximum precipitation takes place between p_H 3·8–4·2. To test the stability to acids and alkalis another sample of purified toxoid (from a different parent toxoid) was adjusted to varying hydrogen ion concentrations. These samples were then, without any re-adjustment of p_H, tested for flocculating value (*a*) immediately, and (*b*) after 72 hours. The results are collected in Table VII.

Table VII. *The effect of different hydrogen ion concentrations on the Lf value of purified toxoids.*

	Tested immediately after adjustment of p_H			Tested after 3 days at 4°		
Sample	p_H	Lf units per cc.	Tf in mins.	p_H	Lf units per cc.	Tf in mins.
A	3·0	No flocc.		3·0	No flocc.	
B	4·4	Precipitation level		Precipitation level		
C	5·0	12	50	5·0	12	75
D	6·0	14	75	6·0	14	135
F	7·0	14	75	7·3*	14	105
Control	7·4	14	70	7·4	14	70
H	8·0	14	75	7·1*	14	100
K	9·0	14	135	7·8*	14	105
L	10·0	14	3 days	8·6*	14	1 day

* p_H changes due to atmospheric effect on solutions of low buffer content.

The solutions at p_H below the acid precipitation zone showed no flocculating value. At all hydrogen ion concentration levels which were definitely above the precipitation zone, however, flocculating values similar to those of the control purified toxoid were obtained. At as high a p_H as 10·0 the value remained the same, although the time of flocculation was considerably increased. This is rather a remarkable result in view of our old ideas with regard to the instability to acids and alkalis of unpurified toxin preserved with phenol. Moloney and Weld [1925] using unpurified toxoid at p_H 10·0 were unable to obtain any flocculation with antitoxins.

The stability to shaking. During the preliminary survey of the chemical properties of purified toxoid the effect of shaking on the degree and time of flocculation with antitoxin was investigated and Table VIII gives some of the results obtained.

Table VIII. *The stability of purified toxoid to mechanical shaking.*

Purified toxoid	Shaken with	Original Lf	Period of shaking (hours)	Lf after shaking
C 4/26	Glass beads	33–34	3	33–34
C 4/20	„	7	3	5–6
C 4/22 A	„	18	3	14–15
C 4/18	Glass beads	14	3	14 (40 secs. TF)
C 4/18	0·5 % phenol + glass beads	14	3	12 (1 day TF)
C 4/18	0·5 g. charcoal + glass beads	14	3	1 (1 day TF)

25 cc. of the purified solutions were shaken vigorously in a mechanical shaker for 3 hours with and without the addition of phenol and charcoal. To ensure uniform shaking the same number of small glass beads were placed in each bottle containing the mixtures. Shaking alone may slightly affect the flocculating value but no appreciable diminutions have been observed. Shaking with 0·5 % pure phenol produces a slight drop in the flocculating value and the time of flocculation is considerably increased. That the active principle is adsorbed by charcoal is shown by the drop in the Lf value from 14 units to 1 unit after 3 hours' shaking.

The stability to rapid aeration.

That solutions of purified toxoid are also comparatively stable to mild oxidation is shown by the fact that if a rapid current of air or oxygen is passed at room temperature through such solutions containing a small amount of capryl alcohol (to reduce frothing) for several hours, there is no loss of flocculating value. At temperatures approximating to 50° destruction is facilitated although the degree of destruction varies with purified toxoids prepared from different parent toxoids.

The chemical properties of purified toxoids.

Culture filtrates prepared from media made by the tryptic digestion of horse muscle consist of muscle extractives and breakdown products of the muscle split off by the action of the enzyme. An analysis of the nitrogenous

components of one of these culture filtrates by the method of Wasteneys and Borsook [1924] showed the following results:

	Mg./10 cc.	% total N
Total N	34·00	—
Protein N	0·07	0·021
Metaprotein N	2·28	6·7
Proteose N	11·59	34·1
Peptone N	15·2	44·7
Sub-peptone N	0·025	0·07
Residual N	4·835	14·409

A large proportion of the nitrogen is thus present as metaprotein, proteose and peptone.

During the growth of the diphtheria bacillus, various nitrogenous changes take place. There is an increase in the nitrogen precipitable by acetic acid and in the Van Slyke nitrogen [Watson and Wallace, 1924, 1]. Very little is known of the action of formalin in the production of diphtheria toxoid. There is a progressive decrease in free amino-nitrogen as the action proceeds and the toxicity of the solution to guinea-pigs steadily diminishes although the flocculating value with antitoxin remains the same. Formaldehyde can always be detected in the final atoxic antigens [Rimini-Schryver method, Schryver, 1910]. Diphtheria toxoid is, therefore, a very complex solution. Although the nitrogenous active fraction precipitable by acetic acid from the toxoid still contains a proportion of extraneous components, a survey may be included here of some of its chemical properties. After dialysis this active fraction gives the same reactions as before, but the colour tests are somewhat less marked. Table IX summarises the chief chemical reactions which survive the purification process.

Table IX. *Some chemical reactions of diphtheria toxoids.*

Test	Diphtheria toxoid	Purified toxoid
For elements		
Nitrogen	+	+
Sulphur	+	+
Phosphorus	+	*
Iron		*
For proteins		
Xanthoproteic	+	+
Hopkins-Cole	+	*
Millon	+	+
Molisch	+	*
Biuret	+	+
For Carbohydrates		
Fehling before hydrolysis	−	−
Fehling after HCl hydrolysis	−	−
For formaldehyde		
Rimini-Schryver	+	+

* In some cases negative results were obtained, in others the test was positive.

As Moloney and Weld [1925] have shown, sulphur survives this purification although whether it is an essential constituent of the toxoid molecule is not known. The fact, however, is interesting in view of Davis and Ferry's work

[1919] on the importance of the sulphur-containing amino-acid cystine for toxin production. Phosphorus apparently is not an essential constituent and the iron is probably derived wholly or in part from the centrifuge used for the concentration. It is invariably present if the Sharples machine is used for this purpose. The active fraction gives most of the usual protein reactions, although contrary to the findings of Moloney and Weld [1925] not all of our purified toxoids give a tryptophan reaction. Formaldehyde is always detectable in the purified toxoid and is, moreover, somewhat difficult to remove even by dialysis.

90 % or more of the total solids from the purified solutions are combustible. No quantitative determinations of carbon, nitrogen, etc. have yet been made. A small percentage of amino-nitrogen can always be detected in the purified solutions and 60–85 % of the total nitrogen is precipitable by Hedin's tannic acid method for the determination of proteose nitrogen.

Summary.

1. The active principle of toxic filtrates of *C. diphtheriae* is confined to a very small fraction of the filtrates.

2. By formalising the filtrates and treating them with acetic acid, a highly active fraction is precipitated.

3. This active fraction can be further purified by reprecipitation methods or by dialysis, and solutions as much as 100 times or more less impure than the original toxoid obtained.

4. Purified toxoid, like diphtheria toxoid, is a very stable substance.

5. The purified solutions are protein in nature and contain sulphur and occasionally phosphorus.

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