LXII. A NOTE ON THE AMMONIUM SULPHATE PRECIPITATION OF THE ACTIVE PRINCIPLE OF THE CULTURE FILTRATES OF *C. DIPHTHERIAE*.

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IN previous papers [Watson and Wallace, 1924, 1, 2; Watson and Langstaff, 1926] the methods of preparation and some properties of the acid-precipitable specific principle of culture filtrates of the diphtheria bacillus have been described. The investigation has been extended to the examination of the precipitates produced by various concentrations of ammonium sulphate, the activity of the precipitates being again estimated by the *in vitro* flocculation test of Ramon [1922]. Throughout the paper the term "active principle" has been used to denote the substance (or substances) present in culture filtrates of *C. diphtheriae* which gives this specific *in vitro* flocculation with antitoxin. While it is probable that these flocculation values are by themselves rarely a reliable index of the antigenic value of the solutions, they obviously afford a valuable method of estimating the combining power of solutions obtained by chemical methods of purification of culture filtrates.

Heineman [1908] utilised the ammonium sulphate method for concentrating diphtheria toxin, and in one case obtained a solution ten times as strong as the original filtrate. Some loss of specific principle—as measured by animal tests (guinea-pig M.L.D.)—always occurred. Seibert and Long [1926], in a recent investigation of the chemical composition of the active principle of tuberculin, used glacial acetic acid and ammonium sulphate as precipitants. While the precipitation was always incomplete with the former, complete saturation with ammonium sulphate appeared to precipitate the whole of the tuberculin activity and the authors advanced experimental evidence to show that the active principle was a whole protein.

EXPERIMENTAL.

The solutions of the crude active material were prepared, except where otherwise stated, by inoculating media made by the three-day tryptic digestion of horse muscle [Watson and Langstaff, 1927] with P.W. 8 strain of *C. diphtheriae* and growing for ten days in the incubator. The cultures were then treated with 0.5 % toluene, 0.5 % phenol or 0.75 % of neutralised formalin

(40 % formaldehyde), filtered after 24 hours and tested for Lf value¹. The culture filtrates were grouped according to Lf values.

For the removal of ammonium sulphate or acid-precipitable material small volumes were filtered through paper. Where large volumes were being dealt with the precipitates were recovered quantitatively by centrifugation in a Sharples' centrifuge, the bowl of which was provided with a detachable celluloid roll, although it is of interest to note that Siebert and Long found the high specific gravity of their ammonium sulphate-tuberculin mixtures (containing glycerol) precluded this. The acid-precipitable material was dissolved in caustic soda to p_H 8.0 and the sulphate-precipitable material in distilled water, in each case to the original volume or to an aliquot part. The very small amount of ammonium sulphate present in these purified solutions seemed to interfere in no way with the flocculation test with antitoxin, although it was difficult to test solutions containing more than traces of the salt owing to rapid precipitation of the serum proteins. One of the features of the testing of the more concentrated solutions was the extreme rapidity of the flocculations. Very active concentrated solutions frequently flocculate in a few minutes at 37°. On diluting these solutions the time of flocculation gradually increases, and this time is not materially altered whether the diluent is water, tryptic digest broth, muscle extract or a two per cent. solution of peptone. The organic nitrogen of the purified solutions containing small amounts of ammonium sulphate was estimated after removing the ammonia with potassium bicarbonate [see Haslam, 1905].

1. *The precipitation of the active principle at various levels of ammonium sulphate concentration.*

To equal volumes of culture filtrates which had been treated with formalin, readjusted to p_H 8.0, filtered through a Berkefeld candle and incubated for a week at 36°, varying amounts of ammonium sulphate were added and the precipitates produced at the complete, two-thirds, one-half and one-third saturation levels were examined. The mixtures were allowed to stand overnight before filtration. The precipitates were washed with ammonium sulphate solution at the corresponding level and then dissolved in water. Estimations of the organic nitrogen and the specific activity of the solutions were made.

The filtrates from these initial precipitations were brought up to complete saturation by addition of solid ammonium sulphate and the second precipitates were washed, collected, dissolved in water, and tested as before.

It will be seen from Table I that the maximum recovery of the active principle is effected by complete saturation with ammonium sulphate, although similar recoveries can be made by precipitation in two successive

¹ As used in this paper the Lf dose may be defined as that volume of any solution containing the active principle of the culture filtrates of *C. diphtheriae* which is equal to one unit of antitoxin by the flocculation test (for fuller definition and description of the test see Glenny and Okell [1924] and Glenny and Wallace [1925]).

stages as shown. For the toxoid under consideration, however, the most pure active precipitate was obtained by two-thirds saturation—the precipitate containing 162 Lf units per mg. of nitrogen. The original toxoid contained 4 Lf units per mg. of nitrogen, so that a relative nitrogen purification of over 40 was effected.

Table I. *Precipitation of a diphtheria toxoid at various levels of saturation with ammonium sulphate.*

Original toxoid. TME 146 containing 14,000 Lf units per l. and 4 Lf units per mg. N.

Degree of saturation	Initial recovery of Lf units				Recovery of Lf units by completing saturation of filtrates from first precipitate			
	Lf units pptd. from 1 l. toxoid	Recovery %	Organic N pptd. from 1 l. (mg.)	Lf units per mg. N of ppt.	Lf units pptd. from 1 l. toxoid	Recovery %	Organic N pptd. from 1 l. (mg.)	Lf units per mg. N of ppt.
Complete	11,700	84	248	47	—	—	—	—
$\frac{2}{3}$	11,500	82	71	162	200	1	102	2
$\frac{1}{2}$	2,600	19	62	42	9,600	69	172	56
$\frac{1}{3}$	0	0	14	0	11,700	84	382	31

2. *The activity of the precipitates produced from one toxoid, (a) by varying the hydrogen ion concentration with acetic acid, (b) by saturating with ammonium sulphate.*

Experiments were next carried out to compare the efficiency of acetic acid and ammonium sulphate as precipitants. A typical experiment may be described. A volume of diphtheria toxoid was prepared and the acetic acid precipitation zone was determined, as described in a previous paper [Watson and Langstaff, 1926]. The p_H limits of this zone were found to be 3.1 to 4.35. Four litres of the toxoid were then precipitated at each of eight points within this zone and the precipitates recovered quantitatively by means of a Sharples' centrifuge. The total solids, total nitrogen content and the specific activity of the precipitate dissolved in alkali to p_H 8.0 were then estimated. A similar volume of the same toxoid (p_H 7.9) was completely saturated with ammonium sulphate and the activity of the precipitate dissolved in water estimated in the same way. The results are given in Fig. 1.

By varying the hydrogen ion concentration of the toxoid, maximum precipitation was produced at p_H 3.9, the isoelectric point of the active "protein" (Curve A). Correlated with this, the precipitate at this point was most active (Curve B). Curve C shows the concentration of Lf units per 20 mg. nitrogen of the precipitates produced at various points in the p_H zone 3.1–4.35. The maximum activity was 4100 units per 20 mg. nitrogen, and since the original toxoid contained 37 units per 20 mg. nitrogen a relative purification of 110 was effected by precipitation at the isoelectric point. Even at this point, however, only 39 % of the total active principle was recovered.

On the other hand, by completely saturating the toxoid (p_H 7.9) with ammonium sulphate, the whole of the activity was recovered in the precipitate (Curve D). As Seibert and Long [1926] have found with tuberculin, ammonium

sulphate is a far more energetic precipitant than acetic acid even at the isoelectric point. In fact a very strong resemblance (apart from heat stability) between the behaviour of the active principle of tuberculin and a true bacterial toxin such as diphtheria toxin may eventually be proved to exist.

Fig. 1. The precipitability of a diphtheria toxoid by change of p_H with acetic acid and by ammonium sulphate.

Curve A. Total solids precipitated from 1 l. of toxoid at various c_H .

Curve B. Specific activity (Lf units) of various precipitates.

Curve C. Lf units per 20 mg. N of the precipitates.

Curve D. Specific activity (Lf units) of the precipitate produced by complete saturation of the toxoid (p_H 7.9) with ammonium sulphate.

3. *The influence of the preservative used for culture filtrates on the precipitability of the active principle.*

The particular chemical agent used for the destruction of the bacilli at the end of growth and the preservation of the culture filtrates during subsequent manipulation may exert a definite influence on the recovery of the active principle by precipitation with ammonium sulphate. The chief agents employed for this purpose are phenol, formaldehyde and toluene, but of these, toluene is the only one that apparently has no action on the active principle. Table II shows the recoveries from a series of culture filtrates treated with different preservatives.

In all cases except two (TB 182 and TX 179—beef-heart peptone media) the media were prepared by the three-day tryptic digestion of horse muscle [Watson and Langstaff, 1927]. In the case of the filtrates treated with phenol, very little of the active principle is found in the precipitate, although examination of the amounts of nitrogen in the material precipitated in the case of unpreserved, toluene- or phenol-treated culture filtrates gave the same results. These low recoveries from phenol-treated filtrates may be due to the "salting out" of the antiseptic and the high concentration localised in the precipitate effecting severe destruction of the active principle [see Pope, 1927]. The

recoveries from unpreserved or toluene-treated filtrates are uniformly high and in some cases practically complete. On the other hand, the formalin-treated filtrates fall into two groups, one giving practically complete recovery and the other comparatively small. In every case where the yields were high the parent toxoid was well matured—*i.e.* the formalin had been in contact with the culture filtrates for a prolonged period and the amount of free formalin had been reduced to a minimum. Further, the p_H of the toxoid had been readjusted to 8.0. The low recoveries were produced from culture filtrates which had been in contact with formalin for a short time only. It would appear that the contributing cause to the low recoveries in these cases is a p_H effect. This effect is examined in the next section.

Table II. *The effect of the presence of preservative on the activity of the precipitate produced by complete saturation with ammonium sulphate.*

No.	Preservative	No. of Lf units per 100 cc.	Lf units recovered by complete saturation with $(NH_4)_2SO_4$	Percentage yield
TME 172	Formalin	1100	1050	96
TME 93	"	425	425	100
CES 171 A	"	1150	550	48*
TME 150	"	1050	1000	95
TTE 154	Toluene	850	750	88
CES 169 A 3	"	1900	1800	95
TTE 181	"	1400	1200	86
TE 178 A	Phenol	1400	350	25
TE 177	"	1600	500	31
TB 182	"	1100	200	18
TX 179	Unpreserved	1200	1200	100
"	Toluene	1200	1200	100
"	Formalin	1200	600	50*
"	Phenol	1200	200	17

* Formalin added a few hours before saturation with ammonium sulphate.

4. *The influence of the hydrogen ion concentration of the culture filtrates on the "salting out" effects of ammonium sulphate.*

Very few data exist in the literature on the salting out of proteins (except perhaps those of serum) by ammonium sulphate. It is probable that the effects produced with any one protein are characteristic only for that protein. With the object of testing the effect of salting out the *diphtheriae* "proteins" in acid and alkaline solutions, a series of culture filtrates were adjusted to different p_H and then saturated with ammonium sulphate. The activity of the precipitates was tested in the usual way. The results are collected in Fig. 2.

It will be seen that complete recovery of the active principle is usually found if the salting out is conducted in slightly alkaline solution (p_H 8.0). At points on the other side of neutrality the recovery may not be complete. In the acid precipitation zone (p_H *ca.* 4.0) very low recoveries are always experienced. This is probably caused by the partial acid precipitation of the

active principle in a form insoluble in water¹. The whole mechanism of the salting out of proteins is an obscure one but it is of importance that the recovery by precipitation with ammonium sulphate of the active principle of diphtheria toxin in a form soluble in water is only complete in the absence of certain chemical substances (*e.g.* phenol or acid) which in some way affect the structure of the active principle.

Fig. 2. The influence of p_H on the precipitation of culture filtrates by ammonium sulphate.

- · · · · Culture filtrate untreated.
- Culture filtrate treated with 0.5 % toluene.
- Culture filtrate treated with 0.75 % formalin.

5. The chemical "purity" of the precipitates produced by acetic acid and ammonium sulphate.

In any work involving the isolation and examination of substances of physiological interest it is obviously an advantage to utilise, as a basis for attacking the problem, material as free as possible from extraneous components. To obtain some idea of the relative purity of the precipitates during the course of the investigation, the concentrations of Lf units per mg. N of the original culture filtrates and the various precipitates have been determined. Different culture filtrates vary widely in their behaviour towards the same precipitant, but the general impression received during the course of the work was that although ammonium sulphate under certain conditions may completely precipitate the active principle from the culture filtrates, the precipitates produced in the acid precipitation zone contain relatively more Lf units per mg. N. The complete examination of a low value culture filtrate is shown in Table III. Acetic acid at the isoelectric point (p_H 3.9) produced a precipitate

¹ Mastin and Schryver [1926] have recently described a well-defined acidic denatured product of albumin soluble in dilute alkalis but insoluble in dilute acids.

which was 110.8 times as "pure" as the original toxoid. The "purest" precipitate obtained in the ammonium sulphate series was that produced by two-thirds saturation where a relative purity of 28.8 was obtained (compare Table I, where the precipitate produced at this level also contained the highest concentration of Lf units per mg. N).

Table III. *The relative purity of the acid- and the ammonium sulphate-precipitable material.*

Toxoid TME 93. 1.85 Lf units per mg. N

Precipitate in zone p_H 3.1-4.35			Ammonium sulphate precipitates		
p_H of pptn.	Lf units per mg. N pptd.	Relative purification of act. principle	Degree of saturation	Lf units per mg. N pptd.	Relative purification of act. principle
4.35	150	81.1	Complete ↓ ↓ ↓ ↓ ↓ ↓		
4.1	132	71.4		17.7	9.6
3.9	205	110.8		47.2	25.5
3.8	163	88		53.2	28.8
3.7	180	97.3		27.2	14.7
3.45	132	71.3		No ppt.	—
3.3	132	71.3			
3.1	74	40			

6. *The chemical properties of the active substances.*

Apart from the presence of small traces of ammonium sulphate and of a larger amount of extraneous material, the chemical properties of the active substances obtained by salting out the culture filtrates are essentially similar to those of the acid-precipitable material previously described [Watson and Langstaff, 1926]. The salted out "proteins" can be freed from ammonium sulphate by dialysis, the loss of active principle depending largely on the nature of the membrane employed. The active material gives typical protein colour reactions and the specific substance is comparatively stable.

SUMMARY.

1. The substance which causes the specific *in vitro* flocculation with antitoxin can be salted out of culture filtrates of *C. diphtheriae* by complete saturation with ammonium sulphate. Recovery is complete in the case of filtrates untreated with any chemical agent, and adjusted to p_H 8.0 before precipitation.

2. The presence of certain chemical agents (*e.g.* phenol or acid) prevents the complete recovery of the active principle by precipitation with ammonium sulphate.

3. The material salted out by ammonium sulphate is less chemically "pure" relative to the original culture filtrate than the acid precipitate produced at the isoelectric point although, in general, the chemical properties of the precipitates are the same.

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