THE PRECIPITATION OF *B. WELCHII* TOXIN.¹

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INTRODUCTION.

THE toxin of *B. welchii*, as it is obtained in filtrates from meat-broth cultures of the organism, differs from the toxins produced by *B. diphtheriæ* and *B. tetani* in two important particulars:—

1. When *B. welchii* is grown under favourable conditions, the toxin which is produced reaches its maximal value in young cultures twelve to twenty-four hours old. After this period, the toxin as a rule depreciates very rapidly in titre, and on the second or third day of incubation it may disappear entirely. Further, if a toxin-containing filtrate be kept at room temperature for one or two days, it may be found to lose 50 per cent. or more of its initial value. The same process of apparent degeneration has been observed to occur in the case of toxins stored in the cold room, although, under these conditions, the rate of disappearance is much more gradual.

2. The potency of *B. welchii* toxin is, to a very marked degree, less than that of diphtheria or tetanus. A good *B. welchii* toxin is one that contains no more than 5- to 10-mouse minimal lethal doses per cubic centimetre.

Both these features, namely, instability and low potency, were found to militate against the carrying out of any extended experimental investigation on *B. welchii* toxin. It was deemed necessary, therefore, to get the toxin in a more stable form, and in a form in which one could conveniently handle a high multiple of the minimal lethal dose. The experiments dealt with in this report were undertaken with this particular object in view. The dried toxin we have succeeded in obtaining by various methods of precipitation has proved to be quite stable. There is thus provided a simple method of measuring the value of sera containing antitoxin to *B. welchii*. Further, the substitution of a concentrated toxin in place of the low value culture filtrates hitherto employed in the immunisation of horses offers the chance of securing, in the future, antitoxic sera of much higher titre than those as yet obtained.

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EXPERIMENTAL DATA.

A. *Precipitation by Ammonium Sulphate.*

Cultures of different toxin-producing strains of *B. welchii*, varying in bulk from 8 to 24 litres, were incubated for eighteen to twenty-four hours, and then filtered, firstly, through layers of paper pulp and sand, and then through large Berkefeld candles under pressure. To the resulting filtrate were added measured quantities of ammonium sulphate. The mixtures were stirred vigorously and then left at room temperature overnight. The precipitate which forms as a cake on the surface of the fluid was then removed. It was freed as far as possible from excess of fluid by kneading in sterile linen cloths, or by squeezing between sterile glass slabs. The sticky mass of precipitate was then chopped into small pieces, which were soaked in repeated changes of absolute alcohol, and finally dried over sulphuric acid. The pieces were then reduced to powder in a mortar, the final product being kept for a further period over sulphuric acid. Finally, the precipitate was accurately weighed, and its toxicity was estimated by determining the minimal lethal dose of solutions of known strength made up with physiological salt solution.

Experiment 1.

FULL SATURATION WITH AMMONIUM SULPHATE.

TABLE I.—PRECIPITATES OBTAINED BY SATURATION WITH AMMONIUM SULPHATE.

TOXIN.	STRAIN.	AGE OF CULTURE IN HOURS.	YIELD OF PRECIPITATE. GRMS. PER LITRE.	TOXICITY OF PRECIPITATE IN MICE.			
				MILLIGRAMMES.			
				5·0	2·5	2·0	1·0
327 A	617 D	20	9·39	++		++	--
" B	"	"	9·36	++		++	--
" C	"	"	8·44	++		++	--
" D	"	"	9·02	++		--	--
328 A	SR 9	22	5·70	++	+-	+-	--
" B	"	"	5·43	++	++	--	--
" C	"	"	5·54	++	++	+-	--
" D	"	"	5·63	++	+-	++	--
329 A	SR 9	40	4·86	++	+-	--	--
" B	"	"	4·88	++	+-	+-	+-
" C	"	"	4·31	++	+-	--	--
" D	"	"	4·46	++	+-	--	--
330 A	SR 9	18	4·34	--		--	--
" B	"	44	4·46	--		--	--
" C	"	18	4·38	+-		--	--
" D	"	44	4·64	--		--	--
331 A	Keenan	20	4·64	++		+-	--
" B	"	"	5·32	++		++	+-
" C	"	"	3·86	++		--	--
" D	"	"	4·96	++		++	--

+ = Death of mouse in 48 hours.

- = Recovery.

The filtrates used in this experiment were obtained from meat-broth cultures of varying age contained in 4-litre bottles. The numbers, 327 to 331, refer to different batches of meat broth, the bottles of each batch being divided into four groups, A, B, C, and D. To bottles marked A and B there was added after autoclaving 0·25 per cent. sterile sodium phosphate. Bottles marked C contained twice this amount of phosphate, while bottles marked D contained no added phosphate. The inoculation in each case consisted in the introduction into each bottle of a small piece of infected pectoral muscle taken from a pigeon which had succumbed to infection by the strain of *B. welchii* indicated. The yield of precipitate is expressed in grammes per litre of filtrate. The toxicity of each precipitate was estimated roughly by the intramuscular inoculation of 1 per cent. solution into mice of 20 grms. weight. Reference to Table I. will show that the majority of these precipitates killed in a dose of 5 mgrms., a few in a dose of 2 mgrms., but none killed consistently in a dose of 1 mgrm. It was found possible to make saline solutions of these precipitates up to 1 in 20, but higher concentrations proved to be viscid and difficult to handle with accuracy. A 1 in 20 concentration of a toxin killing in a minimal dose of 2 mgrms. yields a solution which contains twenty-five lethal doses per cubic centimetre. In other words, saturation with ammonium sulphate produced a precipitate which was too bulky. Moreover, the total yield of toxin in two of the best precipitates proved to be no more than 35 per cent. and 44 per cent. of the amount present in the original crude filtrate.

Experiment 2.

PRECIPITATION BY THE ADDITION OF 500 GRMS. OF AMMONIUM SULPHATE TO A LITRE OF FILTRATE.

TABLE II.—TOXINS PRECIPITATED BY $(\text{NH}_4)_2\text{SO}_4$, 500 GRMS. ADDED TO 1000 C.C. TOXIN.

TOXIN.			PRECIPITATE.		
No.	Minimal Lethal Dose. C.c.	Acidity.	Total Yield.	Minimal Lethal Dose. Mgrms.	Toxin recovered.
358 (1)	0·3	4·5 %	1·91	2·5	38·2 %
358 (2)		2·0 %	1·72	4·0	21·5 %
359 (1)	0·5	5·0 %	2·71	1·5	54·2 %
359 (2)		2·0 %	2·28	2·5	27·3 %
378 (1)	1·0	5·6 %	1·09	2·5	43·6 %
378 (2)			1·31	2·5	52·4 %

Ammonium sulphate, added to filtrate in the proportion of 500 grms. per litre, produces a precipitate, which, as in Experiment I., collects on the surface of the fluid as a scum or cake that can be easily handled.

From filtrate 358, which gave a reaction of 4·5 per cent. acidity when tested with phenol-phthalein, there was obtained a precipitate containing 38·2 per cent. of the total toxin present. The same filtrate reduced to an acidity of 2·0 per cent. yielded no more than 21·5 per cent. of the original toxin, although the total bulk of the precipitate was not very markedly altered. Approximately the same result was obtained with filtrate 359. In both instances, the yield of toxin from the crude filtrate was double that obtained from the same filtrate after its reaction had been reduced to 2·0 per cent. acidity.

This method, then, provided a precipitate which was much less bulky than

that obtained with full saturation, and the toxin content of the precipitate was higher than in Experiment I.

Experiment 3.

PRECIPITATION OF TOXIN BY THE ADDITION OF AN EQUAL VOLUME OF SATURATED AMMONIUM SULPHATE SOLUTION.

TABLE III.—*B. WELCHII* TOXINS PRECIPITATED BY SATURATED $(\text{NH}_4)_2\text{SO}_4$ SOLUTION 1:1.

TOXIN.		PRECIPITATE.			
No.	Minimal Lethal Dose. C.c.	Yield.	Minimal Lethal Dose. Mgrms.	Total Minimal Lethal Doses.	Recovery %.
345 (4)	1.0	0.95	1.4	677	67.7
346 (6)	0.4	2.86	4.0	715	28.6
347 (2)	0.6	3.12	6.0	520	31.3
357 (4)	1.0	0.72	1.4	514	51.4
357 (5)	1.0	0.57	1.0	570	57.0

An equal volume of saturated ammonium sulphate solution was added to measured quantities of filtrate, the mixture being shaken vigorously and left to stand at room temperature overnight. The fine flocculent precipitate was separated off by filtration through hard filter papers. These were dried over sulphuric acid, and the adherent precipitate scraped off and collected. The yields proved to be much better in toxin content than those obtained in Experiment I., but the time involved in filtering the fine flocculent precipitate showed this method to be both tedious and inconvenient.

B. Precipitation by Alcohol.

TABLE IV.—PRECIPITATION OF CRUDE *B. WELCHII* TOXIN BY ALCOHOL.

TOXIN.	MINIMAL LETHAL DOSE.	PRECIPITATION.			PRECIPITATE.			
	Per Litre.	Alcohol.	Vols.	Hrs.	Yield. Grms.	Minimal Lethal Dose. Mgrms.	Total Minimal Lethal Doses.	Recovery %.
345 (1)	1000	Abs.	2	18	1.24	3.0	410	41
345 (2)	„	„	4	18	8.12	10.0	812	81
347 (3)	1660	Meth.	1	18	2.41	6.0	402	24
347 (4)	„	„	2	18	3.20	3.5	811	49
346 (2)	2500	„	2	18	2.59	2.0	1290	50
346 (5)	„	„	2	18	2.47	2.0	1230	49
346 (1)	„	„	2	$\frac{1}{2}$	1.55	1.0	1550	62

The addition to filtrate of alcohol either as absolute alcohol or as methylated spirit results in a fine flocculent precipitate, the sedimentation of which can be hastened by the addition of a trace of ether. In most cases the alcohol filtrate mixtures were left for eighteen hours in the cold room before removal of the precipitate. This prolonged contact with alcohol was not found to interfere in any way with the solubility of the final product in salt solution. A reference to the experimental data set forth in Table IV. shows that 1 volume of alcohol precipitated out about a quarter of the toxin present. Two volumes yielded a precipitate containing half the toxin, while 4 volumes gave three-quarters or more of the toxin.

The precipitates obtained by further addition of alcohol to mixtures from which a 4-volume alcohol deposit had been already removed were found to contain no toxin.

*C. Primary Precipitation by Ammonium Sulphate, combined with
Secondary Precipitation by Alcohol.*

TABLE V.—AMMONIUM SULPHATE PRECIPITATE, 327 C, REPRECIPITATED
WITH ABSOLUTE ALCOHOL.

	Solution.	Dissolved in	Volumes Absolute Alcohol.	Yield. Grms.
F	1 : 10	Water	1	0·28
G	1 : 10	„	1	0·26
C	1 : 50	0·9 salt	3	0·29
L	1 : 50	Water	4	0·36
M	1 : 50	„	4	0·37
E	1 : 100	„	4	0·33
D	1 : 100	0·45 salt	4	0·30
B	1 : 100	0·9 „	4	0·53
X	1 : 100	0·9 „	4	0·47

A toxin, 327 C, obtained in Experiment 1, was chosen for these observations. Amounts of 1 gram. of this toxin were dissolved in distilled water or salt, so as to give solutions of the concentrations indicated. To these solutions absolute alcohol was added in the amounts shown, and the resulting precipitate was filtered off immediately. In bulk, these alcohol precipitates varied from about a quarter to a half of the original. The toxin content of each of these precipitates was tested by determining the minimal lethal dose of a 1 per cent. solution, both for 20 gram. mice intramuscularly, and for full-grown rabbits intravenously. The results are set out in Tables VI. and VII. The amount of toxin recovered in each case, as estimated on

the intramuscular lethal dose in mice and the intravenous dose in rabbits, is represented in Table VIII.

TABLE VIII.—TOXIN RECOVERED IN SECONDARY ALCOHOL PRECIPITATES, AS ESTIMATED ON MOUSE INTRAMUSCULAR DOSE AND RABBIT INTRAVENOUS DOSE.

	Mouse.	Rabbit.
F	70%	66%
G	—	46%
C	58%	67%
L	—	85%
M	74%	66%
E	82%	100%
D	75%	100%
B	66%	—
X	94%	84%

It will be seen that in a favourable experiment the bulk of the original ammonium sulphate precipitate can be reduced to about one-third by secondary precipitation with alcohol, and, further, that the whole of the toxin may be recovered in this secondary precipitation.

DISCUSSION OF RESULTS.

The ideal to be arrived at in precipitating any toxin from solution consists in obtaining a product with the minimum of bulk and the maximum of toxicity. The greater the toxin content of the initial culture filtrate, the more easily is this object obtained. Most toxin-producing strains of *B. welchii*, when grown in meat broth, give at their best a toxin with a lethal dose for mice of 0.1 c.c., so that the total amount of toxin recoverable from a litre represents no more than 10,000 mouse minimal lethal doses. Further, toxin production by *B. welchii* is associated very regularly, though not invariably, with the formation of a sticky substance which is readily precipitated along with the toxin, particularly when ammonium sulphate is used as the precipitant. The ammonium sulphate precipitate, when redissolved, gives solutions that become more and more viscid as the concentration increases. As a rule, solutions of 1 in 100 and 1 in 50 can be easily handled, but solutions of 1 in 20 or 1 in 10 are often extremely difficult to deal with. On the other hand, precipitates obtained by adding one to four volumes of alcohol dissolve very readily even in high concentrations. They also give a more certain and better yield of toxin. Where, however, operations have to be conducted on a

large scale, the expenditure in alcohol becomes very considerable, and it was for this reason that we devised the method outlined in Experiment 5, namely, a double precipitation, firstly by ammonium sulphate, and secondly by alcohol. This method has been used by us on a large scale; the secondary precipitation by alcohol being carried out on solutions of precipitates obtained by two-thirds ammonium sulphate saturation. It is possible by this method to get solutions containing from 50 to 250 mouse minimal lethal doses per 1 c.c. The possibility of getting *B. welchii* toxin in a still more concentrated form would seem to depend entirely on the problem of obtaining better-value culture filtrates.

Our specimens of toxin have been stored at room temperature in amber-coloured bottles fitted with rubber corks, and have been found to suffer no deterioration over a period of eleven months.