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SEDIMENTATION OF BACTERIA WITH POLYETHYLENE GLYCOL

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ABSTRACT

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Experiments are reported which show that the addition of 4,0% polyethylene glycol (6 000 MW) to liquid cultures of *Clostridium welchii* Type D, *Pasteurella* and *Vibrio fetus* (venerialis) will effectively sediment the bacteria. Live *Brucella abortus* S19, a smooth *S. gallinarum* Strain 28 600 and a formalin inactiviated suspension of *V. fetus* (intestinalis) Strain 661 were, however, not sedimented.

When effective the method has appreciable application in the production of vaccines and other processes requiring the easy collection of large volumes of cells, and the separation of bacteria from their soluble exotoxins.

INTRODUCTION

The application of polyethylene glycol (PEG) for the sedimentation and purification of viruses has been well documented (Polson & Deeks, 1963; Mikhailovsky, Tsaing & Atanasiu, 1971; Cook, Babiuk & Hudson, 1972) and the principle has also been used for the same purposes with proteins (Polson, Potgieter, Largier, Mears & Joubert, 1964). The kinetics of the process have been studied by Juckes (1971) on a variety of proteins and brome grass mosaic virus.

In the light of the above knowledge investigations were undertaken to determine whether PEG could also be employed for the purification of a bacterial toxin and sedimentation of bacteria.

EXPERIMENTS

A. *Clarification of* Clostridium welchii *Type D epsilon toxin*

Batches of toxin were produced in an 800 l fermentation tank essentially as described by Jansen (1967).

Experiment 1

Aliquots of culture were treated with different concentrations of PEG* as follows: The pH of the samples was adjusted to 7,0 and dry PEG added to give the desired concentrations. The samples were then shaken until all the PEG had dissolved and left at room temperature overnight. After this period the samples were examined visually and the Lf of the supernatant fluid determined (Jansen, 1967).

TABLE 1 Clarification of *Cl. welchii* Type D epsilon toxin

Aliquot No.	Final concentration of PEG %	Appearance of supernatant fluid	Lf/ml of supernatant fluid
1..........	0	very cloudy	375
2..........	2,0	cloudy	375
3..........	3,0	slightly cloudy	375
4..........	4,0	clear	375
5..........	6,0	clear	300
6..........	11,0	clear	300
7..........	16,0	clear	180

From the results shown in Table 1 it is evident that a concentration of 4% PEG effectively sediments the bacteria in the culture but leaves the toxin concentration of the supernatant fluid unaffected. Subsequent observations have shown that overnight standing is unnecessary and the complete sedimentation of bacteria is effected within 1 h.

Experiment 2

The following experiment was done to find whether PEG sedimentation would deleteriously affect subsequent trypsinization and toxoiding. A number of batches of toxin were therefore prepared, clarified by the addition of 4% PEG as described above and finally trypsin activated and toxoided with formalin as described by Jansen (1967). The results are shown in Table 2.

TABLE 2 Effect of PEG clarification on subsequent toxoiding of *Cl. welchii* Type D epsilon toxin

Batch No.	Lf/ml of original toxin	Lf/ml after PEG clarification, activation and toxoiding
1............	406	125
2............	437	437
3............	343	343
4............	437	343
5............	375	250
6............	437	312

The differences observed in the Lf values of the toxins and the Lf values of the corresponding toxoids are commonly encountered and it can be concluded that PEG treatment does not interfere with the process of toxoiding. It has also been found that toxoids thus prepared are readily precipitated with potassium alum and are suitable for preparing stable water-in-oil emulsions.

B. *Sedimentation of dead bacteria*

Concentration of polyvalent Pasteurella *vaccine*

Polyvalent *Pasteurella* vaccine was prepared and its potency assayed in mice as described by Cameron & Smit (1970). After inactivation with formalin, the desired concentration of 0,5% packed cells was obtained from the bacterial suspension by sedimentation with 4% PEG, and the final vaccine was precipitated with 1% alum.

The results of the potency assay are shown in Table 3. The level of immunity was equivalent to that obtained previously with vaccines not prepared by PEG concentration (Cameron & Smit, 1970). It can therefore be concluded that the immunogenicity is unaffected by PEG.

* PEG (6 000 MW) obtained from Hoechst Pharmaceuticals was used in all the experiments

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TABLE 3 Potency assay of *Pasteurella* vaccine concentrated with PEG

Dilution of challenge suspension	Immunized mice		Non-immunized mice	
	Challenged with *P. multocida* A14g	Challenged with *P. multocida* DI	Challenged with *P. multocida* A14g	Challenged with *P. multocida* DI
	Survivors/10	Survivors/10	Survivors/10	Survivors/10
10^{-1}........	6	9	0	0
10^{-2}........	10	8	0	0
10^{-3}........	10	9	0	0
10^{-4}........	10	8	4	1
10^{-5}........	10	10	9	6

Concentration of Vibrio fetus *vaccine*

Liquid cultures of *V. fetus* (venerialis) were prepared in static Pevitski flasks (Cameron & Brett, unpublished data, 1974) and inactivated by the addition of 0,3% formalin. The yield from the flasks was pooled and divided into 2 aliquots of 5 l each. A 50% solution of PEG was prepared in saline and a sufficient volume of this solution was added to 1 of the flasks to give a final concentration of 4%. After standing at room temperature for 48 h the bacteria in the flask containing PEG had sedimented while those in the untreated flask remained in homogeneous suspension. The clear supernatant fluid was carefully decanted and sedimentation was allowed to continue for a further 48 h, then the supernatant fluid was again removed. The final cell density was measured by means of Hopkin's tubes and when compared with the density of the original culture a 10-fold increase was observed. Whereas the density of a well grown culture is usually in the order of 0,3–0,4% packed cells (Cameron & Brett, unpublished data, 1974) a concentration of up to 4,5% packed cells could be achieved by PEG sedimentation.

An oil emulsion vaccine can readily be prepared from such dense suspensions and has been shown to induce a high degree of immunity in cattle (Schutte, uupublished data, 1974). Further concentration to 10% can easily be achieved by centrifugation, thus further reducing the volume of the vaccine required for effective immunization.

Similar experiments using *V. fetus* (intestinalis) Strain 661 were disappointing since this strain did not sediment in the presence of PEG. However, if a culture of this strain was mixed in equal proportions with a culture of *V. fetus* (venerialis) the mixture of the two organisms could be effectively sedimented with PEG.

C. *Sedimentation of live bacteria*

As in the case of inactivated bacterial vaccines, a high concentration of cells is also required for live vaccines for lyophilization purposes. In the case of *Brucella abortus* S19 a dense suspension of bacteria can readily be obtained by sedimentation with carboxymethyl cellulose (CMC) without loss of viability, but this procedure has not been successful with bacteria belonging to a number of other genera which have been tested (Horwell, 1967). It was therefore of interest to investigate the effect of PEG on *Brucella* and other bacteria which are used for the production of live vaccines.

Sedimentation of B. abortus S19

A dense culture of *B. abortus* S19 was produced in shake cultures as described by Van Drimmelen (1956). Aliquots (200 ml) were placed in measuring cylinders and treated with either 0,06% CMC or 4% PEG and allowed to sediment at 4° C for 72 h. The supernatant 180 ml of fluid was removed from each cylinder and the number of viable bacteria in the remaining 20 ml was determined by plate counts. The results are given in Table 4.

TABLE 4 Comparison of sedimentation of *B. abortus* S19 by CMC and PEG

Cylinder No.	Treatment	Visual observation of sedimentation	Viable bacteria/ml × 10^{10} (average of 3 counts)
1............	0,06% CMC	††††	95,3
2............	4,0% PEG	†††	30,7
3............	None	†	33,7

†††† = Complete sedimentation with clear supernatant fluid
††† = Good sedimentation but supernatant slightly cloudy
† = Very little sedimentation

Although visual observation suggested that PEG gave a satisfactory degree of sedimentation, this was not reflected in the counts. A similar experiment was therefore done in an attempt to obtain better sedimentation by varying the pH and concentration of PEG. Essentially the same results were obtained (Table 5). Once again viability counts were low despite the dense sedimentation, which can be observed visually. No increase in the concentration of viable bacteria is achieved and it appears that many bacteria die during the process. It is therefore clear that PEG is not a satisfactory substance for the sedimentation of live *B. abortus* S19.

Sedimentation of Salmonella *strains*

Broth cultures were prepared from the following rough strains: *Salmonella dublin* 1/17 (Botes, 1964); *S. typhimurium* 34× (Botes, 1964), *S. gallinarum* 5503 (Cameron, Fuls & Van Reenen, 1972) and a smooth virulent strain of *S. gallinarum* 28 600 which is used for preparation of BWD antigen.

The cultures of the strains were each divided into 3 aliquots of 1 l in measuring cylinders and treated as follows:

(a) One aliquot was kept at 4° C overnight, centrifuged and the sedimented bacteria suspended in 100 ml medium.
(b) The second aliquot was kept at 4° C overnight and 900 ml of the supernatant fluid discarded.
(c) Dry PEG was added to the third aliquot to give a final concentration of 4%. The culture was allowed to sediment out at 4° C overnight and the supernatant fluid (900 ml) was then carefully decanted.

The number of viable bacteria in each of the 100 ml samples which were obtained was determined by means of plate counts.

From the results shown in Table 6a two aspects are clear. With respect to the rough strains it is apparent that sedimentation with PEG has no advantage since these strains settle out on their own accord. Although there was little difference between PEG and spontaneous sedimentation after standing overnight there

is a marked difference in the speed at which the process takes place. By visual observation it could be seen that in the case of the PEG aliquots sedimentation was almost complete within 3–4 h thus facilitating the speed with which the material can be processed. In the case of *S. dublin* 1/17 the final recovery was only ±30% of the yield obtained by centrifugation while in the case of *S. typhimurium* 34× it was ±53% and in *S. gallinarum* 5503 ±100%.

TABLE 5 Sedimentation of live *B. abortus* S19 with different concentrations of PEG at various pH levels

pH	PEG concentration %	Visual observation of sedimentation	Viable bacteria/ml × 10^{10}
5	0	–	C
5	2	*	19,1
5	3	***	12,5
5	4	****	15,1
5	5	****	22,8
6	0	–	13,6
6	2	****	11,6
6	3	****	19,1
6	4	****	C
6	5	****	13,1
7	0	–	18,2
7	2	****	14,8
7	3	****	17,0
7	4	****	20,0
7	5	****	C
8	0	–	8,5
8	2	****	15,0
8	3	****	5,4
8	4	****	8,8
8	5	****	6,5

C = Contaminated
**** = Complete sedimentation with clear supernatant fluid
*** = Good sedimentation but supernatant fluid slightly cloudy
* = Very little sedimentation
– = No sedimentation

With respect to the smooth *S. gallinarum* 28 600 strain sedimentation with PEG was ineffective and the organism also did not settle out spontaneously.

TABLE 6a Yield of viable Salmonellae obtained by PEG and other methods of sedimentation

Strain	Viable bacteria × 10^9/ml obtained by		
	Centrifugation	Standing overnight	PEG
S. dublin 1/17 (rough)	30,9	10,1	10,5
S. typhimurium 34× (rough)	17,0	13,0	9,0
S. gallinarum 5503 (rough)	5,7	7,0	6,0
S. gallinarum 28600 (smooth)	13,6	2,0	3,0

The above findings were confirmed in a second experiment using only Strains *S. dublin* 1/17 and *S. gallinarum* 28 600 and comparing the packed cell volume (pcv) as well as the concentration of viable bacteria in the sediments. PEG sedimentation had no advantage in the case of *S. dublin* 1/17 and was ineffective with *S. gallinarum* 28 600 (Table 6b).

TABLE 6b Comparison of packed cell volume (pcv) and viability count of *Salmonella* strains sedimented by different methods

Strain	*S. dublin* 1/17 (rough)	*S. gallinarum* 28 600 (smooth)
Viable bacteria × 10^8/ml obtained by centrifugation × 10^8/ml	30,7	33,0
pcv %	2,0	2,0
Viable bacteria × 10^8/ml obtained after standing overnight	10,9	8,5
pcv %	1,0	0,5
Viable bacteria obtained by PEG sedimentation × 10^8/ml	10,8	7,6
pcv %	1,0	0,4

Apart from a moderate drop in the total yield, the viability of the organisms was neither affected in these experiments nor in numerous subsequent observations made during routine vaccine production. It has also been repeatedly found that the presence of PEG does not affect subsequent lyophilization. (Smit & Visser, unpublished observations, 1974).

CONCLUSIONS

The experiments reported here show that certain bacteria can be effectively sedimented from a liquid culture by the addition of 4% PEG. This procedure proved to be particularly useful in clarifying *Cl. welchii* Type D cultures. The quality of the toxin was unaffected by this process.

Sedimentation of *Pasteurella* and especially *Vibrio* cultures with PEG greatly facilitated the preparation of vaccines in which a high density of these organisms is required. Immunity experiments proved that the potency of both the *Pasteurella* vaccine (Cameron, unpublished data, 1974) and the *Vibrio* vaccine (Schutte, unpublished data, 1974) was as good as that of vaccines prepared by conventional concentration procedures, e.g. centrifugation.

Live bacteria such as *B. abortus* S19 were not effectively sedimented and the process had no particular advantage with respect to live rough cultures of *S. dublin, S. typhimurium* and smooth *S. gallinarum*. It can, however, be employed to advantage with the rough *S. gallinarum* Strain 5503.

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