STUDIES IN ANAPHYLAXIS

IV. THE EFFECTS OF ADJUVANTS AND OF HAEMOPHILUS PERTUSSIS ON THE ANTIGENIC PROPERTIES OF BOVINE PLASMA ALBUMIN IN THE MOUSE

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Summary

An optimal shocking dose of 0·05 mg for bovine plasma albumin was found over a wide range of alum-precipitated sensitizing doses.

Haemophilus pertussis, alum, modified Freund's adjuvant, and a mixture of all three were equally effective as adjuvants in enhancing anaphylactic shock.

Under certain conditions, *H. pertussis* was found to have a depressing effect, but otherwise a marked enhancing effect, on anaphylactic shock in actively sensitized mice.

I. Introduction

Alum precipitation of antigen has been widely used as an adjuvant in immunological studies to enhance antibody production, and more recently to enhance anaphylactic response in the mouse (Malkiel, Hargis, and Feinberg 1953; Solotorovsky and Winsten 1953; Fink and Rothlauf 1954). There is, however, little available information on the effect of alum precipitation of the sensitizing antigen on degree of response to the shocking dose. Solotorovsky and Winsten (1953) sensitized mice with alum-precipitated antigen and then examined the effects of three shocking doses—0·25, 0·065, and 0·016 mg bovine plasma albumin. They found 0·25 and 0·065 mg gave more than 50 per cent. mortality and 0·016 mg 40 per cent. mortality. Their results do not indicate an upper inflexion point in the dose response line and it is possible that a higher shocking dose would have given a greater percentage mortality. Using the same antigen, Sobey and Adams (1957) found an optimal shocking dose of about 0·05 mg over a wide range of sensitizing doses, without alum precipitation. The apparent absence of a constant antigen-antibody ratio with respect to shocking dose could not be accounted for. It is possible that without the aid of an adjuvant the range of antibody response was so limited as not to alter the optimal shocking dose sufficiently to be noticed. If the effect of alum precipitation is markedly to increase the antibody response, and thus the amount of available antibody, then, if the optimal shocking dose is at a constant antigen-antibody ratio, the optimal shocking dose would be expected to increase.

Freund's adjuvant has been used by Wheeler, Brandon, and Petrenco (1950) to enhance anaphylactic shock to egg albumin and guinea pig serum in mice. The

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sensitizing and shocking doses used for egg albumin were large, but in spite of this, the incidence of fatal anaphylaxis was raised from 6 without, to 40 per cent. with, adjuvant. Morgan, Sherwood, and Werder (1957), using Freund's adjuvant with bovine plasma albumin, demonstrated that a high percentage mortality could be achieved. Using a shocking dose of 2 mg they obtained 93 per cent. mortality at 49 days after sensitization.

Haemophilus pertussis, when injected together with the sensitizing antigen, is known to enhance anaphylactic response, as originally shown by Malkiel and Hargis (1952). This enhancement is not entirely an adjuvant effect since Pittman and Germuth (1954) and Munoz, Schuchardt, and Verwey (1954) have shown that *pertussis* injected 4 days prior to shocking will enhance passive anaphylaxis.

In the present paper three sets of experiments are described. In the first the relationships of shocking and sensitizing doses, where the sensitizing antigen was alum-precipitated, are examined; in the second, the adjuvant effects of *pertussis*, modified Freund's adjuvant, and alum are compared; and in the third, further experiments on the effect of *pertussis* in passive and active anaphylaxis are carried out.

II. Materials and Methods

(*a*) *Mice*

Female mice weighing about 25 g, from a randomly bred albino stock maintained in this laboratory, were used throughout. The mice were given standard mouse cubes and water *ad lib.*

(*b*) *Antigen*

Bovine plasma albumin (B.P.A.) (Armour fraction V) was used throughout. All shocking doses were administered by the intravenous injection of 0·5 ml of the appropriate amount of antigen made up in 0·9 per cent. saline.

The method of Proom (1943) was followed with respect to alum precipitation. Alum (potassium aluminium sulphate) (2·5 g) was dissolved in 100 ml 1 per cent. B.P.A. and precipitation effected by the slow addition, with stirring, of 1N NaOH to pH 6·8. The precipitate was centrifuged, washed once in 0·9 per cent. saline, and resuspended in 20 ml saline. This suspension was then assumed to contain 50 mg B.P.A. per ml.

Freund's adjuvant could not be obtained commercially and was made up in the following modified form: Lanoline was added to low-viscosity oil (Shell "Risella 17") in the ratio 1 : 4. Dried, killed mycobacteria (a mixture of *phlei* and *butyricum*) was added to give a final concentration of 2 mg/ml. Antigen of the appropriate concentration in 0·9 per cent. saline was added to this in equal volumes and the mixture emulsified in a blendor. The emulsion was found to be stable over a period of weeks.

Phase I *H. pertussis* was obtained from Commonwealth Serum Laboratories, Parkville, Vic., in suspensions at a concentration of 1×10^{12} organisms/ml.

(c) *Preparation of Antiserum*

Sixty mice were given a single injection of 5 mg alum-precipitated B.P.A. intramuscularly and bled 14 days later by the method described by Sobey and

TABLE 1

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 1

Each treatment group is the sum of four observations

Sensitizing Dose (mg)	Times of Testing									
	14 Days					28 Days				
	Shocking Dose (mg)					Shocking Dose (mg)				
	0·1	0·5	2·5	12·5	Total	0·1	0·5	2·5	12·5	Total
0·25	254	168	176	148	746	248	220	148	111	727
1·0	226	232	198	148	804	260	254	170	148	832
4·0	254	226	198	148	826	254	242	226	192	914
16·0	254	260	198	170	882	260	226	260	198	944
Totals	988	886	770	614	3258	1022	942	804	649	3417

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	*F*
Shocking dose (A)	(3)		
Linear	1	9727	98·1***
Remainder	2	65	1
Sensitizing dose (B)	(3)		
Linear	1	2113	21·3***
Remainder	2	25	1
Times of testing (C)	1	198	2
First-order interactions			
A × B	9	100	1
A × C	3	4	1
B × C	3	67	1
Second-order interactions			
A × B × C	9	178	1·8
Error	96	99	

*** $P < 0{\cdot}001$.

Adams (1955). Blood was collected in heparinized tubes for convenience of handling. After centrifugation the clear plasma was stored at −10°C.

(*d*) *Quantitative Score*

The score as described in Part III of this series (Claringbold and Sobey 1958, p. 435) was used throughout.

(*e*) *Experimental Design*

All the experiments described are of factorial design, and since the treatment variables are not constant they are detailed for each experiment in the text.

III. Results

(*a*) *Sensitizing and Shocking Dose Responses where the Sensitizing Antigen was Alum-precipitated*

(i) *Experiment* 1.—Sensitizing and shocking doses were each varied at four levels. The sensitizing dose, given in a single intramuscular injection, was so

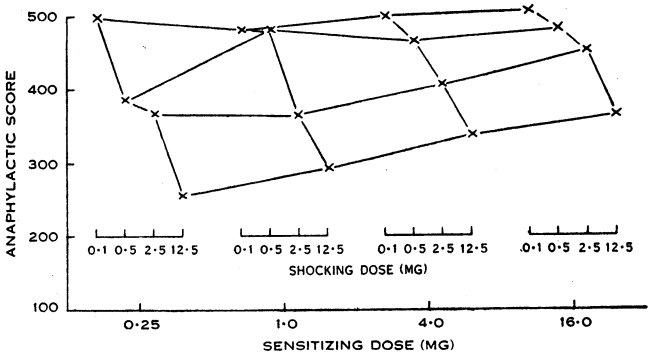

Fig. 1.—Sensitizing and shocking dose responses of experiment 1 plotted as a surface.

scheduled that half the animals were sensitized 14 days, and half 28 days, prior to shocking. This gave a $4^2 \times 2$ factorial design, and with four animals per treatment group 128 animals in all were used. The design, results, and analysis of variance are given in Table 1.

Anaphylactic score decreased log-linearly with increased shocking dose, and increased log-linearly with increased sensitizing dose. The dose responses for both shocking and sensitizing doses are illustrated as a surface in Figure 1, where the data have been summed over the two times of testing (14 and 28 days) since these were not significantly different. There were no significant interactions.

In view of the absence of an inflexion point on the shocking dose response line a further experiment was undertaken with a wider range of shocking dose.

(ii) *Experiment* 2.—Sensitizing and shocking doses were each varied at five levels, and there were five animals per treatment group. This gave a 5^2 factorial design with 125 mice in the experiment. The design, results in full, and analysis of variance are given in Table 2.

Anaphylactic score increased log-linearly with increased sensitizing dose. The shocking dose response line is markedly quadratic with the inflexion point at

TABLE 2

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 2

Sensitizing Dose (mg)	Shocking Dose (mg)					Totals
	0·002	0·01	0·05	0·25	1·25	
0·08	38	38	38	38	38	
	38	38	59	38	0	
	38	59	65	38	38	
	0	59	65	38	38	
	65	38	65	38	38	
Totals	179	232	292	190	152	1045
0·31	0	65	65	38	38	
	29	38	38	65	38	
	29	29	65	65	38	
	65	65	59	38	59	
	38	65	65	38	65	
Totals	161	262	292	244	238	1197
1·25	38	59	38	65	38	
	29	38	65	59	38	
	38	59	65	59	38	
	59	65	65	65	38	
	38	65	65	65	65	
Totals	202	286	298	313	217	1316
5·0	38	65	65	65	38	
	38	65	65	65	38	
	38	65	59	65	38	
	59	65	65	38	38	
	29	38	65	65	38	
Totals	202	298	319	298	190	1307
20·0	29	38	65	65	38	
	38	65	65	65	65	
	38	59	65	65	65	
	29	59	65	38	65	
	29	59	65	65	65	
Totals	163	280	325	298	298	1364
Grand totals ..	907	1358	1526	1343	1095	6229

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	F
Sensitizing dose (A)	(4)		
Linear	1	224	14·45***
Remainder	3	13	<1
Shocking dose (B)	(4)		
Quadratic	1	874	56·41***
Remainder	3	26	1·73
Interactions A × B	16	18	1·18
Error	100	15	

*** $P < 0·001$.

0·05 mg. There were no significant interactions. Sensitizing and shocking dose responses are illustrated as a surface in Figure 2. The data are given in full in Table 2 to indicate clearly the high degree of mortality at the higher sensitizing

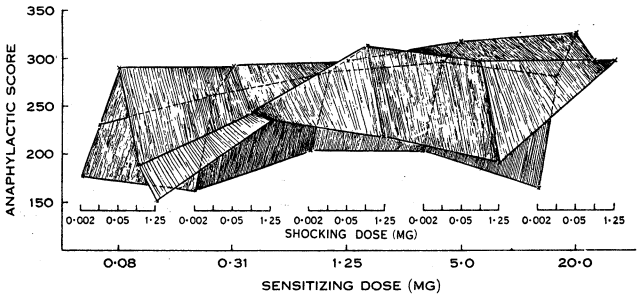

Fig. 2.—Sensitizing and shocking dose responses of experiment 2 plotted as a surface.

doses and to complement Figure 2, in illustrating that while the shocking dose of 0·05 mg is almost uniformly optimal, it is not significantly different from 0·25 and 1·25 mg at the highest sensitizing dose.

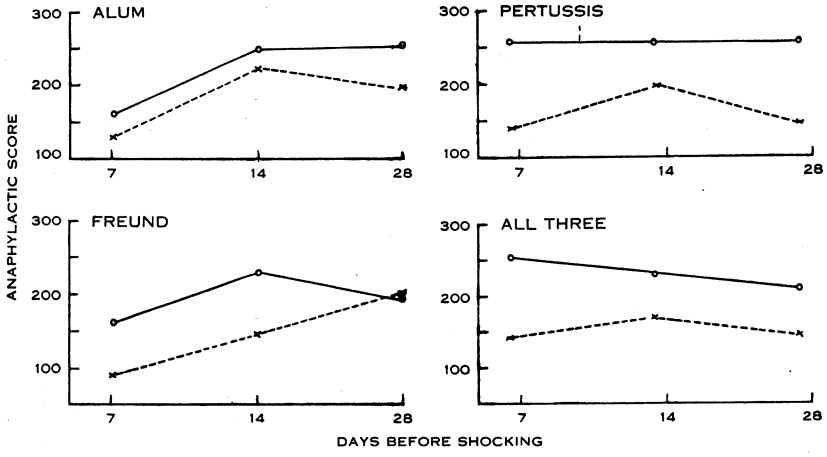

Fig. 3.—Individual dose response lines of the four treatments of experiment 3. Shocking doses: ○ 0·5 mg; × 5 mg.

(*b*) *A Comparison of the Adjuvant Effects of* pertussis, *Modified Freund's Adjuvant, and Alum-precipitated Antigen*

(i) *Experiment* 3.—In this experiment alum, modified Freund's adjuvant, *pertussis*, and a mixture of all three were used as adjuvants. A single 0·1 ml sensitizing injection of each, containing 5 mg B.P.A. made up in the appropriate adjuvant, was administered intramuscularly. Where *pertussis* alone was used the dose consisted of 0·1 ml of a suspension containing 1×10^{12} organisms/ml. The mixture of all three adjuvants thus contained one-third of this amount *pertussis* since it was

TABLE 3

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 3

Each treatment combination is the sum of four observations

Times of Sensitizing prior to Shocking (days)	Shocking Dose (mg)	Adjuvants				Totals
		Alum	Freund	*pertussis*	All Three	
7	5	134	96	141	143	514
	0·05	161	162	260	254	837
		295	258	401	397	1351
14	5	227	152	200	173	752
	0·05	254	233	260	233	980
		481	385	460	406	1732
28	5	200	206	152	152	710
	0·05	260	200	260	206	926
		460	406	412	358	1636
Totals		1236	1049	1273	1161	4719

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	*F*
Adjuvants (A)	3	4,070	2·5
Shocking doses (B)	1	61,281	37·5***
Times of shocking (C)	(2)		
Linear	1	12,692	7·8**
Quadratic	1	11,850	7·2**
First-order interactions			
A × B	3	2,611	1·6
A × B	6	3,587	2·2
B × C	2	1,074	<1
Second-order interactions			
A × B × C	6	1,513	<1
Error	72	1,636	

** $P < 0·01$. *** $P < 0·001$.

made up from an equal volume of each adjuvant. The sensitizing schedule was so arranged that one-third of the mice were injected 7 days, one-third 14 days, and the remaining third 28 days prior to shocking. Two shocking doses, 5 and 0·05 mg,

were used. The experiment was thus a $4\times3\times2$ factorial design and with four mice per treatment group made up a total of 96 mice. The design, results, and analysis of variance are given in Table 3.

There was no significant difference between the four treatments. The scores for the two shocking doses were significantly different, 0·05 mg giving a higher score than 5 mg (see Table 3). Times of sensitizing prior to shocking were significantly different. Response rises to a peak at 14 days and then flattens out. The individual dose-time-response lines for the adjuvant treatments are illustrated in Figure 3. At 7, 14, and 28 days those mice sensitized with *pertussis* as an adjuvant and shocked with 0·05 mg were all fatally shocked.

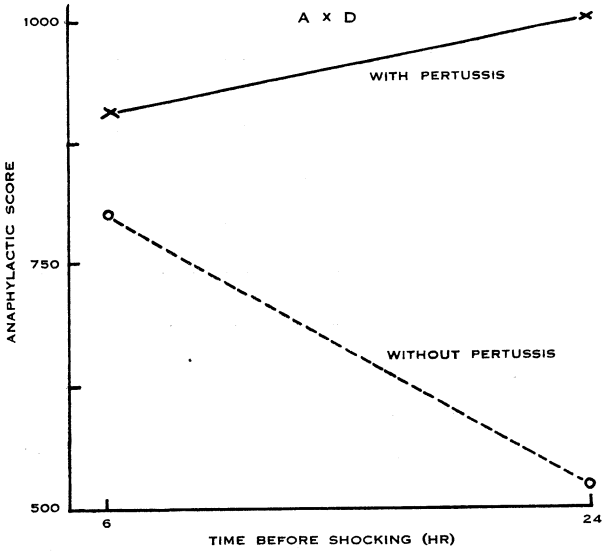

Fig. 4.—Interactions of time between sensitizing and shocking dose and treatment with *pertussis* (A×D) from experiment 4.

(c) *The Effects of* pertussis *under Varied Conditions*

(i) *Experiment* 4.—The effect of *pertussis* in passive anaphylaxis was the main consideration of this experiment. It was of a 2^4 factorial design with four animals per treatment group, i.e. 64 mice in all. The shocking doses were 0·5 and 0·05 mg. Sensitizing doses of 0·5 and 1 ml of mouse anti-B.P.A. were given to half the mice 6 hr, and half 24 hr, prior to shocking. Half the mice were given an intramuscular injection of 0·1 ml *pertussis* suspension containing 1×10^{12} organisms/ml, 4 days prior to shocking. The design, results, and analysis of variance are given in Table 4.

The average score at 6 hr was significantly higher than at 24 hr. Neither the shocking nor sensitizing doses were significantly different in their effect. *pertussis*-treated animals gave a significantly higher score than untreated animals. One first-order interaction, that between *pertussis* and time of shocking (A×D), was significant. This, illustrated in Figure 4, is the result of a marked difference

TABLE 4

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 4

Each treatment group is the sum of four observations

Times of Shocking after Sensitizing	Shocking Dose (mg)	Sensitizing Dose (ml)	With *pertussis*	Without *pertussis*	Totals
6 hr	0·5	0·5	206	221	427
		1	212	179	391
6 hr	0·05	0·5	260	215	475
		1	254	194	448
Totals			932	809	1741
24 hr	0·5	0·5	233	114	347
		1	260	152	412
24 hr	0·05	0·5	260	143	405
		1	260	114	374
Totals			1013	523	1536
Grand totals			1945	1332	3277

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	*F*
Times of shocking (A)	1	863	7·8**
Shocking dose (B)	1	135	1·2
Sensitizing dose (C)	1	0	1
pertussis treatment (D)	1	6460	58·6***
First-order interactions			
A × D	1	1775	16·1***
Remainder	5	108	1
Second-order interactions	4	47	1
Third-order interactions	1	162	1·6
Error	48	110	

** $P < 0·01$. *** $P < 0·001$.

in the slopes of the time response lines. *pertussis*-treated animals gave a higher score at 24 hr than at 6 hr whereas with untreated animals it was the reverse. In the absence of *pertussis*, anaphylactic potential falls off with time from 6 hr after sensitization whereas in the presence of *pertussis* there is an increase in potential with time up to, and possibly beyond, 24 hr. The reason for this is obscure. It

may be due to some interaction of *pertussis* and antibody which requires time to reach its maximal effect.

(ii) *Experiment* 5.—This experiment was a 2^5 factorial design with four mice per treatment group, making up 128 mice. Sensitizing doses of 5 and 0·05 mg and shocking doses of 0·5 and 0·05 mg were used. Half the mice received their last sensitizing injection 7 days, and half 14 days, prior to shocking. The sensitizing dose was given either in a single injection or in two injections spaced by 1 week. All sensitizing injections were given intramuscularly. One week prior to shocking half the mice were injected intraperitoneally with 0·1 ml of a *pertussis* solution containing 18×10^9 organisms/ml. The design, results, and analysis of variance are given in Table 5.

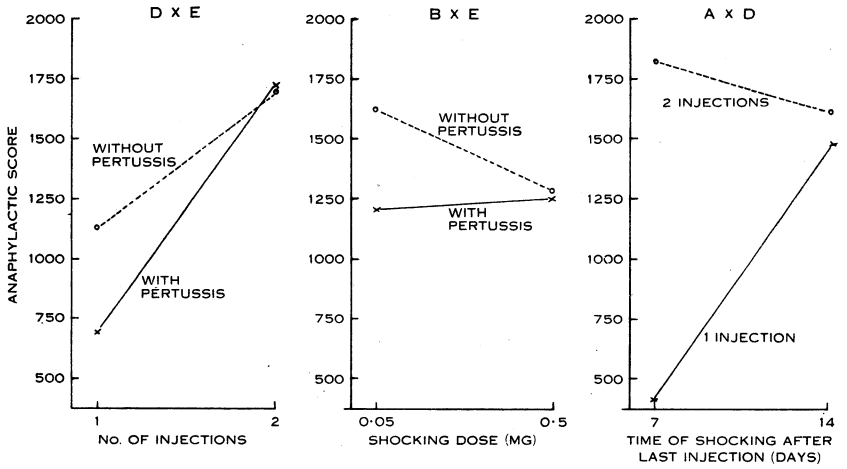

Fig. 5.—Interactions of treatment with *pertussis* and number and level of shocking dose (D×E, B×E) and of number of injections and time between shocking and sensitizing dose (A×D) from experiment 5.

The score at 14 days is significantly higher than at 7 days. Differences between shocking doses, significant at the 5 per cent. level, indicate 0·05 mg to have a higher score than 0·5 mg. While the two levels of sensitizing injection were not significantly different, two injections gave a significantly higher score than a single injection where the same total sensitizing dose was used. Animals given *pertussis* (in this instance given separately from the antigen by the intraperitoneal route) and in lower concentration than in experiment 3, had lower scores than untreated animals. There were two first-order interactions involving *pertussis*: the presence or absence of *pertussis* with the number of injections (D×E), and with shocking dose (B×E). These are illustrated in Figure 5. The first shows that *pertussis* only had a depressing effect on anaphylactic score where a single sensitizing injection was given. This suggests that the depressing effect is associated with the level of antibody response and is only apparent where the response is low. The interaction of *pertussis* with shocking dose indicates that the concentration of the shocking dose is less important in *pertussis*-treated than in untreated animals. The third significant first-order

TABLE 5

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 5

Each treatment combination is the sum of four observations

pertussis	No. of Injections	Sensitizing Dose (mg)	Shocking Dose 7 Days after Sensitizing (mg)		Shocking Dose 14 Days after Sensitizing (mg)		Totals
			0·5	0·05	0·5	0·05	
With	1	5	0	29	170	168	
		0·05	29	29	132	148	
	Total	.. ..	29	58	302	316	705
	2	5	260	214	220	162	
		0·05	204	260	236	192	
	Total	.. ..	464	474	456	354	1748
Totals			493	532	758	670	2453
Without	1	5	74	95	204	254	
		0·05	58	58	176	248	
	Total	.. ..	132	153	380	502	1167
	2	5	220	254	224	254	
		0·05	198	242	132	220	
	Total	.. ..	418	496	356	474	1744
Totals			550	649	736	976	2911
Grand totals			1043	1181	1497	1646	5364

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	*F*
Time of testing (A)	1	6,555	46·3***
Shocking dose (B)	1	657	4·6*
Sensitizing dose (C)	1	450	3·2
No. of injections (D)	1	220,504	144·7***
pertussis (E)	1	1,637	11·5**
First-order interactions			
A × B	1	2	<1
A × C	1	85	<1
A × D	1	14,028	99·0***
A × E	1	95	<1
B × C	1	237	<1·7
B × D	1	53	<1
B × E	1	1,176	8·3**
C × D	1	0	<1
C × E	1	504	3·6
D × E	1	1,697	12·0***
Second-order interactions	10	154	1·1
Error	96	142	

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

interaction was between the number of sensitizing injections and the time of shocking after the last injection (A × D). This suggests that the maximum score can be expected 14 days after the first injection where two injections are given, the second injection being responsible for rise in score at this point but having no marked effect on the time of optimal response.

(iii) *Experiment* 6.—To obtain further information on the effect of pertussis the following experiment was undertaken. It was a $2^2 \times 3$ factorial design with five animals per treatment group, i.e. 60 mice in all were used. The primary aim was to obtain information on the effects of *pertussis* other than those of an adjuvant.

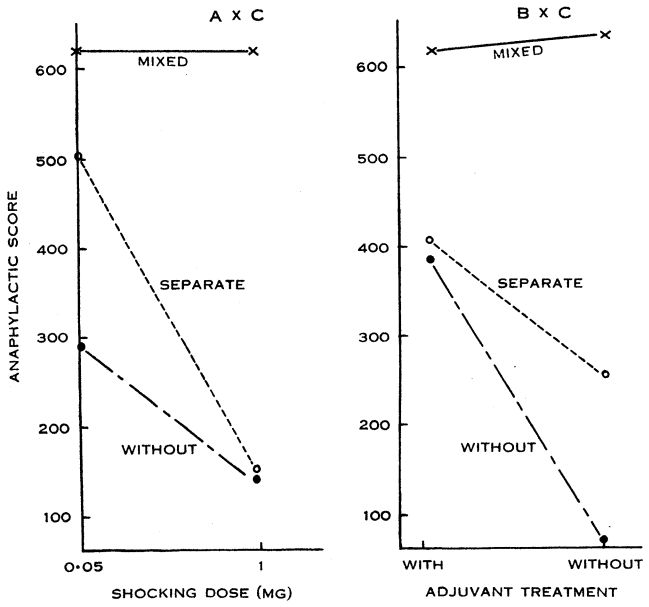

Fig. 6.—Interactions of treatment with *pertussis* and shocking dose (A × C) and of treatment with *pertussis* and adjuvant treatment (B × C) from experiment 6.

Two levels of shocking dose, 1 and 0·05 mg, and two adjuvant treatments, with and without alum precipitation, were used. Three *pertussis* treatments were used: (1) the antigen was mixed with *pertussis* prior to injection; (2) *pertussis* and antigen were injected at different sites (opposite hind legs); (3) no *pertussis* was given. All sensitizing injections were of 5 mg and were administered intramuscularly. For all *pertussis* treatments, 0·1 ml of a suspension containing 1×10^{12} organisms/ml was used. Animals were subjected to shocking doses 14 days after sensitization. The design, results, and analysis of variance are given in Table 6.

The shocking doses were significantly different, 0·05 giving a higher score than 1 mg. Alum-precipitated antigen resulted in a significantly higher score than untreated antigen. The three *pertussis* treatments were significantly different, the highest score being achieved where *pertussis* was mixed; here almost uniform fatality

resulted. *pertussis* given in separate sites gave a higher score than no treatment. The reason for this apparent contradiction of the previous experiment is not clear; it may be associated with the use of a different route of injection, concentration of *pertussis*, and time of administering *pertussis*.

TABLE 6

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 6

Each treatment combination is the sum of five observations

Shocking Dose (mg)	Adjuvant Treatment	*pertussis* Treatment			Totals
		Mixed	Separate	Without	
1	With alum pptn.	319	94	125	538
	Without alum pptn.	319	65	29	413
		638	159	154	951
0·05	With alum pptn.	315	319	269	903
	Without alum pptn.	325	195	29	549
		640	514	298	1452
Totals		1278	673	452	2403

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	*F*
Shocking dose (A)	1	4150	11·9**
Adjuvant (B)	1	3792	10·9**
pertussis (C)	2	9095	26·1***
First-order interactions			
A × B	1	859	2·5
A × C	2	1594	4·6*
B × C	2	1515	4·4*
Second-order interactions			
A × B × C	2	318	<1
Error	48	348	

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

Two first-order interactions involving *pertussis* were significant at the 5 per cent. level, namely *pertussis* × shocking dose (A × C) and *pertussis* × adjuvant treatment (B × C), and these are illustrated in Figure 6. In both instances the interaction appears to be the result of marked differences in the slope of the dose response lines. When mixed with the antigen the *pertussis*-treated mice were all fatally

shocked irrespective of the shocking dose, or whether the antigen was alum-precipitated or not. Animals which were treated with *pertussis* in a separate site from the antigen gave higher scores than untreated animals and were sensitive to the optimal shocking dose (A×C, Fig. 6); they gave a higher score than the untreated animals, where no alum was used.

IV. Discussion

It is clear from our data and that of Solotorovsky and Winsten (1953), Malkiel, Hargis, and Feinberg (1953), Morgan, Sherwood, and Werden (1957), and others that adjuvants, in general, greatly enhance anaphylactic shock. Since adjuvants are known to enhance antibody response it is reasonable to argue that increased anaphylactic potential (Sobey and Adams 1957) is a direct consequence. An optimal shocking dose of 0·05 mg was found over a wide range of alum-precipitated sensitizing doses. The same optimal shocking dose was found by Sobey and Adams (1957) over a wide range of sensitizing doses in the absence of an adjuvant. This strongly suggests that the size of the shocking dose is the important factor and not an optimal antigen-antibody ratio, as might be expected. The reasons for this are at present inexplicable.

Alum precipitation, modified Freund's adjuvant, *pertussis*, and a mixture of all three of these proved to have similar capacities for enhancing anaphylactic shock under the conditions of testing. Morgan, Sherwood, and Werder (1957), using Freund's adjuvant, obtained their highest percentage mortality 35–42 days post-sensitization using a shocking dose of 2 mg B.P.A. In view of this evidence it is probable that the modified Freund's adjuvant would have proved more effective in our tests had they been made over a greater period of time after sensitization. Such long time intervals are often inconvenient. If it is merely desired to obtain a high percentage mortality this can readily be achieved in 7 days using *pertussis*, or 14 days using Freund's adjuvant or alum, provided the optimal shocking dose is used.

When *pertussis* was injected 4 days before shocking in passive anaphylaxis (expt. 4) it markedly increased anaphylactic potential. A similar increase in potential was observed in active anaphylaxis where *pertussis* and antigen were injected at different sites 14 days prior to shocking. These observations suggest that *pertussis* may enhance anaphylactic potential by more than just the enhancing of antibody response. Malkiel (1956) has shown that the effect of *pertussis* is not the result of a direct toxic effect on the adrenal gland. In view of the evidence of Riley (1953) on the histamine content of mast cells, and that of Freund and Stone (1956) on the production of Arthus reaction in the lips of mice and rats, areas rich in mast cells, it is tempting to speculate that the action of *pertussis* may in some way be associated with the sensitivity or multiplication of cells responsible for the production or storage of agents concerned in the shock reaction (histamine, serotonin, etc.). The observation in experiment 5 that *pertussis* does not always enhance, and may even depress, anaphylactic potential emphasizes our lack of knowledge of its mode of action and the need for detailed observations on the effects of dose, route of injection, and time of injection prior to shocking.

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VI. References

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