THE TREATMENT OF CERTAIN EXPERIMENTAL ANAEROBIC INFECTIONS WITH SULPHAPYRIDINE AND WITH IMMUNE SERA AND THE PROBLEM OF SYNERGIC ACTION

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The dramatic success of the sulphonamide series of drugs in the treatment of certain infections caused by aerobic organisms and their use as substitutes for serum therapy has tended to obscure the potential advantages that might accrue from some well-founded scheme of combined treatment with drug and immune serum. Reviews of the experimental data in this field of combined treatment (Long & Bliss, 1939; Fleming, 1939) indicate that complementary or synergic action may be readily demonstrated. We have been interested in the theoretical and practical aspects of the problem of combined action in so far as it might be concerned in the prophylaxis and therapy of infection caused by certain spore-bearing anaerobes.

The suggestion has been made that synergic action when evinced in the combined treatment of certain aerobic infections is due to the bacteriostatic action of the drug supplemented by the agglutinative and tropic effects of the antiserum. Now it is known that immunity to experimental infection with *Vibrion septique* can be effected by active immunization with the bacterial bodies and by passive transference of the antibodies so developed (Robertson & Felix, 1930; Henderson, 1934, 1935, 1937). Craddock & Parish (1931) have also shown that a passive immunity may be conferred on mice against infection with *V. septique* by the administration of relatively large doses of antitoxin. In unpublished experiments one of us (H.) attempted unsuccessfully to demonstrate synergic activity between *V. septique* antitoxin and antibacterial serum. These experiments were by no means exhaustive but the tentative conclusion reached was that no marked complementary action could be demonstrated. Two factors now encouraged us to pursue this problem still further. One was the published evidence which suggested that sulphanilamide or related compounds were of value in the control of experimental gas-gangrene infection (Long & Bliss, 1937, 1939; Buttle, 1939). The other was the advent of the war which gave the whole problem immediate practical significance. It was considered that a study of the combined action of a drug with antibacterial serum or antitoxin might provide a better understanding of the mechanism of synergic action and incidentally increase our knowledge of the pathogenesis of anaerobic infections.

The organism selected for study was *V. septique*. Experimental infection with this organism can be carefully graded and regular results may be obtained with one spore suspension over a period of years; in fact, the suspension used for the most part in this work was prepared in 1937. In addition, clear cut protection experiments with antibacterial serum or antitoxin are readily obtained. On the other hand, some control experiments with *Cl. welchii* seemed to be desirable not only because of its dominant position as a cause of gas gangrene but also because of possible fundamental differences in the mechanism of invasion with this organism as compared with *V. septique*. The work with *Cl. welchii*, however, has been purely of a preliminary nature although the evidence that has accrued is of special interest when contrasted with that obtained in experiments with *V. septique*.

In these experiments we have not been concerned with the assessment of the relative merits of the various chemotherapeutic substances now available. We have selected for trial two compounds only, sulphanilamide and sulphapyridine. The latter drug—M. and B. 693—proved to be unquestionably superior and gave such eminently satisfactory results that experiments on the use of sulphanilamide were suspended indefinitely.

Experimental methods

All experiments have been carried out with mice of mixed breed and of diverse stocks weighing 18–22 g.

The strains of V. septique *and* Cl. welchii *examined.* The classical strain of *V. septique*—Pasteur III—was selected as one that possessed a high degree of virulence for the mouse; it had been used extensively in previous experiments (Henderson, 1934, 1935, 1937). A type "A" strain of *Cl. welchii* (S 107) of human origin was chosen as probably the most suitable one in our collection; this strain is the one used at Elstree for the production of toxin but its power of invading the tissues of the mouse is not great.

The test dose of organisms and the route of infection. For infection with *V. septique* a spore suspension was prepared from a 48 hr. growth on Dorset egg medium, washed in distilled water and then heated for 1 hr. at 75° C. This suspension, which retains its potency for years, is diluted as required with saline, an equal volume of a freshly prepared 5% solution of $CaCl_2$ in distilled water added and 0·1 c.c. of the mixture is injected according to the route selected. The most sensitive tests were carried out by injecting the mixture of spore and $CaCl_2$ intradermally on the shaved abdomen. The test dose was adjusted to contain approximately 100 certain infecting doses (C.I.D.) and of over 300 control mice tested one only survived—a mouse which was probably not injected. The same mixture injected into the muscle of the leg also killed 100% of control mice but on further titration it was not found to be appreciably more lethal when given by this route.

Satisfactory spore suspensions of *Cl. welchii* could not be obtained and the test dose, therefore, was prepared from an 18 hr. meat broth culture. The

required amount was centrifuged and the organisms resuspended to the original volume in 0·85% saline. This suspension was diluted with an equal volume of 5% $CaCl_2$ in distilled water and 0·1 c.c. of the mixture was injected without delay. When given by the intradermal route this dose was only irregularly effective, the death rate in different experiments varying from 50 to 100%; injected intramuscularly, however, this dose regularly killed 100% of mice but did not contain more than 5 certain infecting doses.

The immune sera. Antibacterial serum for *V. septique* was prepared in rabbits according to methods previously described. This serum contained antibody to the O antigen as well as to the labile antigenic component already shown to be of importance in antibacterial immunity (Henderson, 1937); the antitoxic content of the serum was less than 5 units per c.c. *V. septique* antitoxin was prepared in the horse and the concentrated product contained 1900 units per c.c. For experiments with *Cl. welchii* only concentrated antitoxic horse serum containing 3000 units per c.c. was used.

All injections of serum were made intravenously and the dose was contained in 0·5 c.c. of NaCl.

The administration of the drug. Sulphapyridine (M. and B. 693) obtained as a fine white powder was suspended in 0·85% NaCl immediately before use. The dose given at any time rarely exceeded 20 mg.; this amount was contained in 0·5 c.c. NaCl and was given *per os* from a syringe fitted with a wide-gauge blunted needle. Sulphanilamide (Sulphonamide-P Burroughs-Wellcome), when used, was given in the same manner.

RESULTS OF EXPERIMENTS WITH *VIBRION SEPTIQUE*

Prophylaxis and therapy with sulphapyridine. Throughout this work no attempt has been made to find the minimal effective dose of drug that could be used at any one time. We have elected rather to use a "reasonable" dose (20 mg.) which lies well within the limits of toxicity for the mouse and to make the time of administration of this dose the variable factor.

In orientating experiments the test dose of spores (100 C.I.D.) was injected intradermally and the drug was given either before and after infection or after infection only. The results of several of these experiments are summarized in Table 1, from which it will be seen that the survival rate ranged from 74 to 93% according to the system of treatment. Two doses of the drug, one given prophylactically and one therapeutically, saved the highest percentage of animals (93%). If treatment was commenced 4 hr. after infection and further doses of the drug given at the 8th and 20th hour a slight but significantly lower percentage of survivors resulted (85%), whereas one therapeutic dose given 4 hr. after infection saved 75% of animals. These results are the more dramatic when it is known that all control animals were dead 12–18 hr. after infection, whereas deaths in treated animals were delayed mostly until the 2nd, 3rd or 4th day. The mice were observed for 7 days, but under these particular experimental conditions death from gas gangrene rarely if ever occurred after the 4th day.

Table 1. *The influence of sulphapyridine on the course of* Vibrion septique *infection in mice*

Treatment with sulphapyridine	No. tested	Mice		% of survivors
		Survived	Died	
20 mg. 20 hr. before and 4 hr. after infection	43	40	3	93
20 mg. 4, 8 and 20 hr. after infection	20	17	3	85
20 mg. 4 hr. after infection	39	29	10	74
Nil	75	0	75	0

Drug given *per os* in 0·5 c.c. salt solution.
Test dose: 0·05 c.c. spore suspension.
0·05 c.c. 5% solution $CaCl_2$.
0·10 c.c. injected intradermally (approximately 100 certain infecting doses).

Clearly defined skin lesions appear both in treated and in untreated mice. About 1 hr. after infection there is generally a well-marked oedematous swelling about 0·5 cm. in diameter. This area gradually extends and becomes distinctly haemorrhagic about the 6th to 8th hour. Thereafter, in untreated mice the lesion becomes much more markedly oedematous and rapidly extends to cover practically the entire abdominal surface. In some control animals the first signs of illness appear quite suddenly about the 11th or 12th hour and death may then supervene within half an hour. In others, illness may be delayed until about the 18th hour but a characteristic feature in control mice is that death occurs very rapidly after the onset of symptoms. In treated mice, at about the 12th hour, there is evidence that the lesion is becoming definitely circumscribed; it is then about 1·5 cm. in diameter and deeply haemorrhagic but not more obviously oedematous than at the 6th or 8th hour. By the 24th hour the central area, which is necrotic, is bounded by a clearly defined white band of reactive tissue about 2 mm. in diameter. Thereafter, in animals that make a complete recovery an open necrotic sore appears about the 50th hour, after which the lesion rapidly contracts and is completely healed in 7–10 days. In treated mice that die from gas gangrene after the 24th hour there may or may not be evidence of further spread of the lesion. A feature which is in marked contrast with control mice is that illness may be prolonged for 1–3 days by which time the animal is grossly emaciated.

The influence of the time of administration of the drug. In view of the marked activity of sulphapyridine in saving life it was of interest to examine in more detail the influence of the time of administration of the drug on the survival rate. Table 2 summarizes the results of one experiment in which the test dose of spores was given intradermally. The mice were divided into four groups which received respectively one dose of 20 mg. of the drug at the 4th, 5th, 6th and 7th hr. after infection. By delaying for 3 hr. the administration of the drug, i.e. from 4 to 7 hr., the death rate is seen to rise from 40 to 100%. The death time of fatal cases in the four groups is also markedly different, falling

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from 2 to 3 days in the group treated 4 hr. after infection to 12–33 hr. in the one treated at the 7th hour; this latter death time is only slightly in excess of that recorded among control mice. It has been shown above that repeated doses of the drug given to mice first treated 4 hr. after infection results in an increased survival rate. On the other hand, all attempts to influence the course of events in mice first treated 7 hr. after infection have failed; larger or repeated dosage of the drug are without avail even in prolonging the life of the animals. For example, in one experiment two groups of twenty mice each received 20 mg. of the drug 7 hr. after infection and one group received a further 20 mg. at the 12th hour. None of the mice in either group survived and all were dead by the 30th hour. There was, however, some evidence of delay in death over that observed in a group of ten control mice. The survival rate of 60% shown in Table 2 for the small group of mice first treated 4 hr. after infection is significantly lower than that for mice recorded in Table 1, which were similarly treated. Reference will be made later to variation of this kind that we have observed in experiments with large groups of mice and the most probable explanation is to be found in variation of the genetic composition of mice obtained from diverse stocks.

Table 2. *The effect of the time of administration of sulphapyridine on the course of* Vibrion septique *infection in mice*

	Mice			No. of mice found dead at the following times							
Treatment with sulphapyridine	No. tested	Sur-vived	Died	12 hr.	24 hr.	33 hr.	2 days	3 days	4 days	5 days	6 days
20 mg. 4 hr. after infection	10	6	4	0	0	1	2	1	0	0	0
20 mg. 5 hr. after infection	10	5	5	0	1	2	1	1	0	0	0
20 mg. 6 hr. after infection	10	3	7	0	5	1	1	0	0	0	0
20 mg. 7 hr. after infection	10	0	10	2	6	2	—	—	—	—	—
Nil	10	0	10	1	9	—	—	—	—	—	—

100 C.I.D. injected intradermally. Drug given *per os* in 0·5 c.c. salt solution.

The route of spore infection and the effectiveness of sulphapyridine. Earlier work (Henderson, 1935) had shown that the route of infection with *V. septique* spores determined in some degree the protective efficiency of an antibacterial serum. Thus, more serum was required to protect against a test dose given intramuscularly than against a similar dose given intradermally. On the other hand, the intradermal test was much more sensitive and better adapted for work on serological analysis. For this reason, therefore, the intradermal test was used for preliminary experiments with sulphapyridine. From the practical point of view, however, a relevant criticism would be that the initiation of an anaerobic infection rarely, if ever, occurs in skin but nearly always in deep muscle tissue and that for this reason the intradermal test is only of academic interest. It seemed desirable, therefore, to examine the effectiveness of sulphapyridine in the control of intramuscular infection.

The small inoculum of 0·05 c.c. spore suspension plus 0·05 c.c. 5% solution of $CaCl_2$ used for intradermal testing was found to be equally effective if injected intramuscularly in the leg. Further, when given by this route the killing power was not appreciably greater than when given intradermally; the test dose contained approximately 100 C.I.D. Table 3 summarizes the results of intramuscular experiments in which two or more doses of sulphapyridine, each of 20 mg., were given by mouth: the first, 4 hr. after the test dose, the second at the 8th hour, and subsequent doses at the 24th and 32nd hours after infection. The table shows that of 105 mice treated with two doses of the drug only fifteen or approximately 14% survived and of 105 mice given repeated doses approximately 27% recovered. These results form a striking contrast with those obtained in intradermal tests where the survival rate in mice otherwise similarly treated ranged from 75 to 84%.

Table 3. *Illustrating the effect of sulphapyridine on mice injected intramuscularly with* Vibrion septique *spores* + $CaCl_2$

Treatment with sulphapyridine	Experiments 1		2		3		4		5		Total	
	Sur.	Died	Sur.	Died	Sur.	Died	Sur.	Died	Sur.	Died	Sur.	Died
20 mg. 4 and 8 hr. after infection	4	16	0	20	6	19	1	19	4	16	15	90
20 mg. 4, 8, 24, 32 hr. after infection	7	13	2	18	14	11	1	19	4	16	28	77
Nil	0	10	0	10	0	10	0	10	0	10	0	50

Test dose: 0·05 c.c. spore suspension.
0·05 c.c. 5% $CaCl_2$ solution.
0·10 c.c. injected intramuscularly in leg.
Drug given *per os* in 0·5 c.c. salt solution.

The results of experiments shown in Table 3 are recorded separately with the object of demonstrating the degree of variation encountered from one experiment to another. The methods and materials used in the conduct of all experiments were identical and as stated above, the spore suspension is a stable product that retains its potency for years apparently unaltered. The only variable has been the test animal, the genetic composition of which probably accounts for these discrepancies.

The poor results obtained in the therapy of intramuscular infection led to experiments in which a prophylactic dose of the drug was given 4 hr. before infection followed by one or more doses at the 4th, 8th, 24th and 32nd hours after the test dose. By this means the survival rate was raised significantly but not to the same high level obtained in intradermal tests; thus 27% of mice receiving two doses of drug recovered and with repeated dosage there was a 55% recovery. It is clear, therefore, that if survival rate alone is taken as a criterion of effectiveness, the intramuscular test greatly reduces the efficiency of sulphapyridine and, as we have noted above, this is not because the test dose of spores given intramuscularly contains an appreciably greater number of

certain infecting doses than the same dose given intradermally. If, however, in place of survival rate a comparison is made of the death time among treated mice that have been tested intramuscularly with that of those dying from intradermal infection it is seen that the marked delay in death in the early stages of the experiments is closely similar in both groups. Table 4, which illustrates this point, shows the number of mice treated with the drug, dying or found dead at various periods up to 7 days after intradermal or intramuscular infection. In the first place the general prolongation of life over that of controls is clearly demonstrated. Secondly, it will be noted that in the early stages of the experiments the proportion of mice dying at any given time is closely similar in both groups, the highest percentage occurring about the 48th hour. After this time, deaths among mice injected intradermally rapidly fall to zero whereas in those tested intramuscularly a high proportion of deaths occurs at the 56th hour as well as at the 2nd, 3rd and 4th day after infection. It is probable that the natural defence mechanism of the host encounters greater difficulty in mustering an effective barrier in muscle tissue than it does in skin; co-operative action between drug and phagocyte is, therefore, less efficient. Again, the potency of the toxin produced in muscle tissue is probably greater than that elaborated in skin and as is shown below there is no indication that sulphapyridine can neutralize the toxin of *V. septique*.

Table 4. *The influence of sulphapyridine on the "death time" among mice injected intradermally or intramuscularly with* Vibrion septique *spores* + $CaCl_2$

Route of infection	Treatment with sulphapyridine	No. of mice		Mice found dead at the following hours after infection								
		Sur.	Died	24	32	48	56	72	96	120	144	168
Intradermal	20 mg. 4 hr. after infection	29	10	0	1	7	—	2	0	0	0	0
Intramuscular	20 mg. 4 and 8 hr. after infection	5	35	0	2	13	12	6	2	0	0	0

All of twenty control mice injected intramuscularly found dead 17–24 hr. after infection.
All of forty control mice injected intradermally found dead 17–24 hr. after infection.
The same test dose was used for all mice.

The practical point emerging from these experiments is that in the control of experimental muscle wound infection with *V. septique* the drug alone cannot be relied on to save more than a small proportion of animals. Information, therefore, on combined drug and serum treatment seemed essential. Experiments of this kind have been carried out and the results appear later in the text.

The action of sulphapyridine on V. septique *toxin*. The success attending the use of sulphapyridine might, at least in part, be due to its ability to neutralize the exotoxin of this organism. Experiments designed to demonstrate such possible inactivation *in vivo* have, however, been entirely negative. For example, in one experiment forty mice were injected intravenously with an L+/2 toxin-antitoxin mixture. Twenty of these mice received 20 mg. of drug

by mouth 1 hr. previous to injection, the remaining twenty served as controls. Twenty-four hours after receiving the test dose four of the treated mice were dead and five were comatose, whereas, in the control group eight mice were dead and one was very ill. On the second day, however, the number dead in both groups was the same, namely nine. The remaining mice were observed for 4 days by which time none were ill. This slight delay in death time recorded for the treated mice has no statistical significance and most probably is due to slight variation in the condition of the test animals. The L+ method of testing was selected for the special purpose of excluding possible interference from the toxoid present in the test toxin.

Prophylaxis of V. septique *infection with sulphanilamide.* In two preliminary tests with this compound only seven out of forty (17·5%) animals were protected as compared with 73–93% in similar tests with sulphapyridine. A generalization should not be made from these results that sulphanilamide is of little or no value in the control of any type of *V. septique* infection. For example, there is a considerable difference in the rates of absorption and elimination of these two drugs and we may not have obtained optimal conditions for the action of sulphanilamide. Our primary interest was to select for subsequent combination experiments with immune serum, one of the sulphonamides that would give a satisfactory degree of protection; the eminently successful results with sulphapyridine made further experiments with sulphanilamide unnecessary.

The combined action of immune serum and sulphapyridine in the control of *Vibrion septique* infection

To collect evidence on this point we used the more sensitive intradermal method of inducing spore infection. This form of test allows a more accurate assay of the therapeutic reagents to be made and offers better opportunity for observing synergic activity. The design of experiment was to assess for the reagents (drug, antibacterial serum, and antitoxin) the amount of each which, acting alone, would allow of not more than a 5–10% survival rate and then to observe the effect on survival of various possible combinations of the selected doses of drug and immune serum. The experiments were so arranged that the reagents were given the best possible opportunity of acting together at a critical stage in the infective process.

The selected dose of immune serum and sulphapyridine. From earlier unpublished experiments (H.) it was known that pure antibacterial serum would protect mice if given intravenously 7 hr. after infection by the intradermal route; approximately 100% of mice could be saved by this means. As already mentioned the antibacterial serum at our disposal was prepared in the rabbit and contained antibodies to the O antigen as well as to the labile somatic antigen (Henderson, 1937) of the homologous strain used for infection; it contained less than 5 units of antitoxin per c.c. 0·01 c.c. of this serum given intravenously 6 hr. after infection protected 7–8% of mice (see Table 5),

whereas 0·1 c.c. saved approximately 100% of animals. Antitoxin was also known to be of value in the therapy of intradermal infection although never so regular in its action. We have used a concentrated globulin fraction of antitoxic serum prepared in the horse containing 1900 units per c.c.; 40 units of this serum given intravenously 6 hr. after infection protected approximately 8% of mice (see Table 5), but as much as 300 units saved only 60–70%. From Table 2 which records the results of an orientating experiment in the therapeutic use of sulphapyridine, we have seen that one dose of 20 mg. of the drug given 6 hr. after infection saves 30% of mice whereas none are saved by the same dose given 7 hr. after the test dose. The variation from one experiment to another has already been mentioned and in large-scale experiments the proportion of survivors among mice first treated 7 hr. after infection was found to range from 0 to 17%. It is important to note that unlike antibacterial serum or antitoxin larger or repeated doses of the drug given at this time have already been shown to effect no improvement on the survival rate or indeed on the death time. Under the particular conditions of experiment, therefore, the 7th hour after infection apparently registers the time limit of effectiveness for sulphapyridine.

The doses of immune serum and of drug finally selected for use in experiments on combined action were as follows:

Antibacterial serum: 0·01 c.c. intravenously 6 hr. after infection.

Antitoxic serum: 40 units intravenously 6 hr. after infection.

Sulphapyridine: 20 mg. *per os* 7 hr. after infection.

The tests for combined action. The synergic activity of three possible combinations of the therapeutic agents has been examined. Table 5 records the results of six experiments, each with three groups of thirty mice, designed as follows. In each of the first three experiments the combined activity of two agents has been compared with that of the agents acting separately. In the next three experiments the activity of two of the possible combinations has been compared with that of one agent which is common to both. Thus, summing all the experiments ninety mice have been treated with each of the three reagents acting alone and ninety with each of the three combinations of these agents.

We see from Table 5 that the pooled results for the three agents acting separately present a particularly uniform picture; in each instance the survival rate is 7–8%. The degree of variation from one experiment to another has no statistical significance except in those with sulphapyridine where the survival rate ranges from 0% (Exp. 2) to 17% (Exp. 4). It is of interest to note that such variation has never been apparent in experiments with immune sera.

An extremely effective co-operative action between antitoxin and sulphapyridine is clearly demonstrated by the pooled results of the combination tests which show a survival rate of 88%. The analogous combination of drug and antibacterial serum is also highly effective, saving 72% of mice. In one test (Exp. 2) the death rate among mice treated with this combination is parti-

Table 5. *The influence of various combinations of sulphapyridine, antitoxin and antibacterial serum on the course of* Vibrion septique *infection in mice*

	Experiments												Sum of experiments			
	1		2		3		4		5		6					
Mice treated with	Sur.	Died	Sur.	Died	Sur.	Died	Sur.	Died	Sur.	Died	Sur.	Died	Tested	Sur.	Died	% surviving
Sulphapyridine	2	28	0	30	—	—	5	25	—	—	—	—	90	7	83	7·7
Antitoxin	2	27	—	—	3	27	—	—	2	28	—	—	89	7	82	7·8
Antibacterial serum	—	—	1	29	3	27	—	—	—	—	3	27	90	7	83	7·7
Sulphapyridine plus antitoxin	26	4	—	—	—	—	28	1	25	5	—	—	89	79	10	88·7
Sulphapyridine plus antibacterial serum	—	—	12	18	—	—	26	4	—	—	27	3	90	65	25	72·2
Antitoxin plus antibacterial serum	—	—	—	—	6	24	—	—	2	28	6	24	90	14	76	15·5

Test dose of spores $+CaCl_2$ injected intradermally.

Table 6. *The synergic action of antitoxin and sulphapyridine in the control of intramuscular infection with* Vibrion septique

Therapeutic agent	Time of administration after test dose	Mice		No. of mice found dead at the following hours after test dose										
		Sur.	Died	17	24	28	33	48	56	72	96	120	144	168
Sulphapyridine (each dose 20 mg. *per os*)	4 and 8 hr.	4	16	0	0	0	1	3	5	5	2	0	0	0
	4, 8, 24, 32 hr.	4	16	0	0	0	1	3	3	5	4	0	0	0
Antitoxin (100 units intravenously)	6 hr.	0	20	2	2	10	3	3	—	—	—	—	—	—
Sulphapyridine (20 mg. per dose) plus antitoxin (100 units intravenously)	Drug—4 and 8 hr. Serum—6 hr.	8	12	0	0	0	0	1	1	6	4	0	0	0
	Drug—4, 8, 24, 32 hr. Serum—6 hr.	14	6	0	0	0	0	0	0	0	5	1	0	0
Nil	—	0	10	10	—	—	—	—	—	—	—	—	—	—

cularly high but this is not maintained in the other two tests (Exps. 4 and 6). In striking contrast with these results the combination experiments with antibacterial serum and antitoxin show an almost complete absence of co-operative action. A survival rate of 15·5% in these latter tests compares very unfavourably with the 88% survival in experiments with antitoxin and sulphapyridine.

A theoretical consideration of the results shown in Table 5 will be given later in this paper but in summary it may be said that synergic activity between antitoxic or antibacterial serum and sulphapyridine is clearly established. On the other hand, there is no evidence of synergic action between antitoxin and antibacterial serum, and indeed, as will be shown, they probably act quite independently.

The synergic action of antitoxin and sulphapyridine in the control of intramuscular infection. The relative inefficiency of the drug for the control of intramuscular infection has been recorded in the earlier experiments and it seemed advisable to examine the synergic activity of antitoxin and sulphapyridine in mice infected by this route. Preliminary tests showed that the activity of antitoxin, like that of the drug or antibacterial serum, was much reduced when the test dose was given intramuscularly. Whereas 200–300 units of antitoxin given intravenously 6 hr. after intradermal infection would protect 60–70% of mice, the same amount would protect only about 5–10% of mice when tested intramuscularly. It was apparent, therefore, that the proposed tests of combined action were to be particularly severe and certain orientating experiments showed that a single administration of drug together with one of serum was quite insufficient. If, however, repeated doses of the drug were given to mice that also received one dose of antitoxin then a marked synergic activity was evident. Table VI records the results of one experiment which demonstrates this action. It will be seen that 100 units of antitoxin failed to protect any mice although an extension in death time over that of control mice is clearly shown. Two therapeutic doses of sulphapyridine protected 20% of mice. This appears, however, to register the limit of effectiveness of the drug because it is seen that if the treatment is continued until the 32nd hour after infection the survival rate is not improved. On the other hand, the combined action of two doses of drug with one of antitoxin raises the survival rate to 40% and in the group receiving serum together with continued drug treatment, 70% of mice survive. The difference in the survival rate in these last two groups is of special interest in view of the fact that continued treatment with drug alone failed to effect any improvement over that registered in mice treated with two doses of drug only. A possible explanation of this result is that toxin elaborated in the early stages of the infective process predetermines the death of the animal; continued bacteriostatic action, therefore, is almost valueless. In the presence of antitoxin, however, this continued check to bacterial development allows the natural defences of the host opportunity to take possession of the invader. Support for this reasoning

may be obtained from a perusal of the death times among mice in the various groups recorded in Table 6. It will be seen that treatment with drug alone exerts a marked delay in death over that of either control mice or those treated with antitoxin alone. On the other hand, there is no appreciable difference in the death time between the group receiving two doses of drug alone and the one treated up to the 32nd hour after infection. There is, however, a further obvious delay in death in the "combined" groups, the one receiving continued drug treatment being definitely the better.

Results of experiments with *Cl. welchii*

It is notoriously difficult to secure regular experimental infection with *Cl. welchii*. Satisfactory spore suspensions have not so far been obtained and one is obliged to use vegetative forms of the organism. Frequently also, either massive doses of the bacilli have to be given in order to get regular deaths in control animals, or the strain produces such a potent toxin that the animals die within a few hours of receiving the test dose from an acute toxaemia and before there has been any opportunity for extensive bacterial invasion of the host tissues.

In earlier unpublished experiments (H.) great difficulty had been experienced in protecting mice against *Cl. welchii* infection with immune serum prepared by immunizing with the bacterial bodies. Preliminary experiments have been confined, therefore, to the use of sulphapyridine (or sulphanilamide) and to *Cl. welchii* antitoxin.

The marked success of sulphapyridine in the control of intradermal infection with *V. septique* was not maintained in experiments with the particular strain of *Cl. welchii* that had been selected and sulphanilamide proved to be even less effective. For example, if the drug were first given after infection no evidence of its activity could be demonstrated. Prophylactic treatment seemed to be essential but even here the results were disappointing. Table 7 records a series of small-scale experiments in which a prophylactic dose of 20 mg. of the drug was given 1 hr. before an intradermal infection, followed

Table 7. *The influence of sulphapyridine on the course of* Cl. welchii *infection in mice*

Treatment with sulphapyridine	Experiments 1 Sur.	1 Died	2 Sur.	2 Died	3 Sur.	3 Died	4 Sur.	4 Died	5 Sur.	5 Died	Total no. Sur.	Total no. Died
20 mg. 1 hr. before test dose; 20 mg. 4 hr. after test dose	10	0	2	8	2	18	6	4	7	13	27	43
Nil (controls)	5	5	0	10	1	19	2	8	3	17	11	59

Test dose: 0·05 c.c. washed suspension of vegetative bacilli.
0·05 c.c. 5% $CaCl_2$ solution.
0·10 c.c. injected intradermally.
Drug given *per os* in 0·5 c.c. salt solution.

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by a similar dose 4 hr. after the test dose. The infecting suspension, prepared according to the method described above, was extremely irregular in its action from one experiment to another, the death rate among control animals varying from 50–100% and it is important to note that in the absence of $CaCl_2$ the vegetative bacilli failed to establish infection. The cause of this irregularity is not known but again, a contributory factor is probably the variable genetic composition of the mice. In this connexion it is interesting that in experiments with *V. septique* such variation is only seen in treated animals, whereas regularity in death among control mice is a constant feature.

Despite these irregularities the pooled results given in Table 7 offer clear evidence that this strain of *Cl. welchii* is susceptible to the action of sulphapyridine. Thus, of seventy control mice, 15% survived and of seventy treated mice, 40% survived. This result, which has a high level of statistical significance, is further substantiated by the fact that the death time among control mice was 18–48 hr., whereas in treated mice, death was frequently delayed until the 3rd or 4th day. Attempts were made to improve upon these results by giving large doses of the drug prophylactically or by extending the treatment after infection; these procedures were without avail and in fact there was some indication that continued treatment introduced a "depression" effect. On this latter point no statistical proof can be offered but, if substantiated, it would obviously have an important practical bearing.

The intramuscular injection of a test dose similar to that used for intradermal infection regularly killed 100% of mice, but a slight reduction in the number of organisms produced irregular results. Sulphapyridine given prophylactically and/or therapeutically to mice injected by this route failed entirely to save life and only rarely was there evidence of delay in death. As with *V. septique*, therefore, the route of infection with *Cl. welchii* plays an important role in determining the efficacy of the drug. Long & Bliss (1937) injected the test dose of *Cl. welchii* intraperitoneally. We have not tried this most unusual method of inducing anaerobic infection but the success they attribute to sulphanilamide may, apart from strain differences, be due to the route of infection that they elected to use.

In marked contrast with the relative inefficiency of the drug *Cl. welchii* antitoxin was spectacularly successful. A concentrated globulin fraction of an antitoxic serum prepared in the horse has been used throughout. 2·5 units of this serum, which contained 3000 units per c.c. protected 100% of mice, when given intravenously 6 hr. after a test dose injected intramuscularly; 0·1 unit just failed to protect any of the mice. From this evidence it may be inferred that the strain of *Cl. welchii* used for infection was one of low invasive capacity and that neutralization of accumulated toxin made conditions for continued bacterial development impossible. If this reasoning is correct then treatment with sulphapyridine might have been expected to be more successful for, as shown above, the strain is not insusceptible to the action of the drug. It remains, however, that infection with a highly invasive strain of *V. septique* is

more certainly controlled with sulphapyridine than is the infection with this particular strain of *Cl. welchii*. This raises the problem that will be discussed later, of possible differences in the mechanism of invasion in the two organisms.

Experiments on the combined action of antitoxin and drug have not been carried far principally because of the inherent difficulties of infection with *Cl. welchii*. In experiments with *V. septique*, where 100% of control animals died and the survival rate of mice treated with the reagents separately was adjusted to 5–10%, it was considered essential from a statistical point of view to use groups of not less than twenty to thirty mice. If, however, the death rate in control mice is less than 100%, the statistical evaluation of the results becomes extremely difficult and very large groups are required. On the other hand, intramuscular infection with *Cl. welchii* did not appear to be influenced by sulphapyridine, whereas, very careful adjustment of small amounts of antitoxin was necessary in order to avoid too high a survival rate. The evidence obtained, however, is indicative of a positive correlation between antitoxin and drug. Actually in the control of intramuscular infection we have not succeeded in saving life by the combined use of subeffective amounts of serum and drug, but delay in death time has been clearly established. More careful adjustment of the experimental conditions would in all probability yield successful results.

Carpenter & Barbour (1939) have reported that sulphanilamide is capable of neutralizing *Cl. welchii* toxin. We have been unable to demonstrate any neutralizing action on the part of sulphapyridine. The L+ method for testing this point was selected with a view to excluding possible interference with toxoid present in the test toxin. Groups of twenty mice received 20 mg. of drug 1 hr. before the intravenous injection of an L+/5 toxin-antitoxin mixture and the mice were observed for 4 days. There was no significant difference in the survival rate of treated and control mice although there was some slight delay in death time in the groups receiving sulphapyridine.

Discussion

The genesis of infection with a spore-bearing anaerobe is determined by the physico-chemical conditions governing germination of the spores. Fildes (1929*a*, *b*) has shown that the factor determining the germination of these spores is an adequately negative oxidation-reduction potential at a suitable *p*H, the critical potential varying with each species. In natural infection these conditions are fulfilled in areas of trauma where the damaged cells are cut off from the continuous flow of body fluids or where there is an actively growing aerobic organism. Once germination of spores has taken place the course of the disease is determined not only by the products of bacterial metabolism but also by the capacity of the vegetating bacillus to invade the healthy tissues of the host. The species included in the gas-gangrene group of spore-bearing anaerobes vary greatly in this respect. Those of low invasive capacity resemble

in many ways *Cl. tetani*. They may remain relatively localized near the original site of tissue damage, although the necrotizing toxin they elaborate extends potentially the area for their proliferation. Bacteriological records of field investigations into certain animal diseases produced by certain types of *Cl. welchii* (Lamb dysentery and Infectious entero-toxaemia of sheep) or *Cl. oedematiens* ("Big Head" and Infectious necrotic hepatitis of sheep) bear witness to this fact. On the other hand, the truly invasive species such as *V. septique* and *Cl. chauvoei* and possibly also certain strains of *Cl. welchii*, are not confined within the extending area of necrosis and may be found advancing into apparently healthy tissue.

These variable types of infection with spore-bearing anaerobes present difficult problems in immunity. The three lines of approach to prophylaxis or therapy are (1) the control of bacterial proliferation, (2) the neutralization of accumulated toxin or (3) a combination of both. With regard to the first of these methods it has been shown experimentally that infection with highly invasive species such as *V. septique* and *Cl. chauvoei* may be readily and completely prevented by the development of antibacterial immunity (Robertson & Felix; Henderson, *loc. cit.*), and in the field the prophylactic value of *Cl. chauvoei* vaccine is undisputed. This method, however, may be of little or no value in the prevention or treatment of infection with anaerobes of low invasive capacity. For example, in experimental infection with *Cl. tetani*, Russell (1927) has shown that the periphery of a lesion supporting vegetating tetanus bacilli becomes in effect a phagocytic barrier while the area of trauma remains practically free from phagocytes. These non-invasive bacilli therefore, remain in secretive seclusion at the original site of tissue damage, thereby rendering any direct phagocytic attack virtually impossible. This evidence is substantiated by Coleman & Gunnison (1931), who failed to establish passive protection against infection with *Cl. tetani* by means of a pure antibacterial serum. In a series of unpublished experiments (H.) we found that the only condition under which some evidence of antibacterial immunity to tetanus could be demonstrated was where the spores were injected together with large numbers of non-pathogenic vegetating bacilli (e.g. non-toxic *Cl. tetani* or *B. subtilis*); by this means germination of spores was permitted to occur in tissue that was not cut off from the continuous flow of body fluids. The route of infection as well as the conditions permitting germination of the spores probably limits the efficiency of any prophylactic or therapeutic interference. Mayer (1938, 1939) has reported successful results in the chemotherapy of experimental *Cl. tetani* infection with sulphanilamide and related compounds whereas Long & Bliss (1939) state that this drug is without effect on experimental infection with *Cl. tetani*. This discrepancy might well be explained by differences in the experimental methods which in the one case might produce a superficial lesion that gave the chemotherapeutic agent access to the offending organism but in the other might establish a deep necrotic lesion that the drug was unable effectively to penetrate.

The experiments reported in the present paper bring into sharp relief these differences in the invasive properties of different species in the gas-gangrene group as well as the difficulties encountered in the development of antibacterial measures of control. Under certain experimental conditions sulphapyridine, antibacterial serum or antitoxin were highly effective in controlling *V. septique* infection. On the other hand, infection with the particular strain of *Cl. welchii* was remarkably sensitive to treatment with antitoxin but the activity of sulphapyridine was much less marked than in *V. septique* infection; it was clear, however, that *Cl. welchii* was not insensitive to the action of the drug. The difference in the response of the two infections to chemotherapy might imply that the two organisms varied in their sensitivity to the action of the drug, and here it must be emphasized that only one strain of each species was examined. As first suggested by Whitby (1938) it is possible that drug-resistant strains may be encountered. Nevertheless, the marked difference already observed in the response of *V. septique* and *Cl. welchii* infections in general to treatment with sera prepared from the bacterial bodies supports the view that these two organisms differ fundamentally in their modes of attack on the animal body.

The control of infection by the neutralization of accumulating toxin is, in many respects, to be regarded as a secondary line of defence. The theoretical basis on which this method is employed as a single defence system is that the continual neutralization of accumulating toxin allows the animal body to mobilize antibacterial and repair mechanisms that will deal effectively with a traumatic lesion supporting bacterial proliferation. As discussed below, however, the experiments described in the present paper clearly demonstrate that no synergic activity is obtainable with a combination of antitoxic and antibacterial sera; the effectiveness of antitoxic measures alone, therefore, is problematical. For example, it has been noted above that infection with a non-invasive species such as *Cl. tetani* precludes the possibility of an early attack by the natural defence mechanisms of the host. Russell (1927) has shown in experiments with guinea-pigs that sporulation of vegetating tetanus bacilli commences about 5 days after the initial germination of injected spores and it might be supposed that after this time the lesion would become quiescent. One of us (H.) has observed, however, that regermination of newly formed spores is a frequent occurrence in *V. septique* infection and this probably holds true also for tetanus because we have also observed that the number and frequency of the doses of tetanus antitoxin necessary to save life is dependent to a large degree on the severity and size of the original area of trauma. Antitoxic measures for the control of infection with species of high invasive capacity are likewise problematical in their effectiveness. Here again, the final issue is determined by the ability of the host to muster an antibacterial defence and the efficacy of this system, which in such infections is immediately operative, is, as we have seen in the present experiments, greatly influenced by the route of infection.

The problem of synergy. The chief interest in the present study has centred around the possibility of synergic activity on the part of two or more therapeutic agents. From earlier experiments with *V. septique* there was no indication of synergic action on the part of antitoxic and antibacterial serum. Wider possibility for the study of this problem came with the finding of sulphapyridine as a third therapeutic agent. For the control of one infective process it was now possible to examine the combined activity of two agents that would act respectively on two entirely different substances, namely, the invading organism and its potent exotoxin or alternatively, to examine the combined activity of two agents directed against one of these substances, namely the organism.

Before considering the results obtained it is necessary to examine the various ways in which two therapeutic agents might react when administered together. Some workers seem to believe that if there is no synergic action between the two agents and P individuals survive with one and Q with the other, then when they are used together $(P+Q)$ individuals ought to survive; this, however, is extremely unlikely. It would mean that every population must contain P individuals that cannot be saved by one agent but can be saved by the other and vice versa. There is no guarantee that the numbers P and Q are approximately equal and it is difficult to see why the above conditions should be expected to hold true. If two agents are used in subeffective amounts both of which act in a somewhat similar manner (e.g. controlling bacterial proliferation) one might expect that the survivors would be those with the highest natural resistance. In this case, if there is no synergic action, it might be expected that the same individuals would be saved by either agent. If they are used simultaneously, therefore, the survival rate should not be changed much from P or Q but would depend upon which was the greater. A third possibility is that the two agents act quite independently of one another. Thus, if antitoxin and antibacterial serum are used in sub-effective amounts it might be conceived that the individuals who survive are those with the highest natural resistance to toxin and bacterial proliferation respectively and that resistance to these agents is determined quite independently. If this be true and the agents are used simultaneously, the number of individuals surviving would be $(P+Q)-PQ$. Where $P=Q=50\%$ it will be noticed that $(P+Q)-PQ=75\%$, which is much less than $(P+Q)$. On the other hand, if $P=Q=10\%$ then $(P+Q)-PQ=19\%$ which is very nearly equal to the sum. However, if there is synergic action between the two therapeutic agents the survival rate will be greater than $(P+Q)-PQ$, although there is no hypothesis we can construct that will tell how much greater the value will be. The precise statistical tests for the significance of deviations from the above expectations are somewhat complicated and they are being treated by Dr K. Mather in a separate communication.

In the experiments with *V. septique* the dosage of the three reagents (sulphapyridine, antitoxin and antibacterial serum) was so adjusted that when

acting alone they each allowed of a 7–8% survival rate (Table 5). Table 8 summarizes the observed results in the tests for combined action and gives also the values calculated from the formula $(P+Q)-PQ$. From this we see that clear evidence was obtained of synergic activity between sulphapyridine and either antitoxic or antibacterial serum. On the other hand, there is no indication of combined action on the part of antitoxin and antibacterial serum; in fact, the results are in close agreement with the expected value if these agents acted independently.

Table 8. Vibrion septique *infection in mice: observed and expected values in tests for the combined action of sulphapyridine and immune sera*

	Observed results		Expected values	
Treatment	Survived	Died	Survivals	Deaths
Sulphapyridine plus antitoxin	79	10	13·5*	76·5
Sulphapyridine plus antibacterial serum	65	25	13·5	76·5
Antitoxin plus antibacterial serum	14	76	13·5	76·5

The reagents acting separately were tested in groups of ninety mice. Dosage was so adjusted that the survival rate in each group was 7–8% (see Table 5).

Expected values calculated from the formula $(P+Q)-PQ$, where P and Q are the proportions surviving with each agent used separately.

* Value based on a group of ninety mice.

Perhaps the most interesting feature of these results is the marked difference between the combined action of sulphapyridine plus antitoxin and that of antibacterial plus antitoxic serum. The former combination probably works in such a manner that permission for intervention is offered to a third factor, namely the natural defence mechanism of the host. The slight but direct bacteriostatic action of the sulphapyridine and the neutralizing action of the antitoxin gives time for the natural defence system to be mustered. One may deduce, therefore, that it is this third factor which, in the presence of the other two agents, brings about the striking result. On the other hand, in the combined action of antibacterial and antitoxic serum only two factors may operate. In contrast with the specific growth inhibiting action of sulphapyridine the antibacterial serum has only an agglutinative and tropic action. But, as we have seen, this activity alone is insufficient and with the second line of defence—the antitoxin—also present in insufficient amount the result obtained is not unexpected. In the tests with sulphapyridine and antibacterial serum again only two factors are involved. It has been shown that the drug has no neutralizing action on *V. septique* toxin and consequently the combined action of drug and serum falls on one agent in the infective process—the invading organism—apparently with marked success.

This research gives good grounds for believing that chemotherapy may prove a useful adjuvant in the control of certain anaerobic infections in man. Furthermore, it must be borne in mind that such infections are likely to be complicated by the presence of aerobes which may be susceptible to the action

of the sulphonamides. However, it must be emphasized that apart from the necessity for efficient surgical treatment, these drugs used as the sole prophylactic or therapeutic interference cannot be relied upon to control gas-gangrene infection and an attempt should be made to ellicit the synergic effect produced by the combined action of drug and serum.

SUMMARY

1. Sulphapyridine is an efficient prophylactic or therapeutic agent against intradermal infection with the strain of *Vibrion septique* selected for test, whereas, in intramuscular infection the drug is unreliable and saves only a relatively small proportion of animals.

2. The strain of *Cl. welchii* (type A) selected for test is not insusceptible to the action of sulphapyridine. A significant degree of protection against intradermal infection may be obtained however, only when a prophylactic dose of the drug is given. The drug is apparently without action against intramuscular infection with this strain.

3. Under the particular experimental conditions sulphanilamide is much less effective than sulphapyridine against infection with either *V. septique* or *Cl. welchii*.

4. There is no evidence that sulphapyridine given *per os* neutralizes the toxins of *V. septique* or *Cl. welchii* injected intravenously.

5. It is possible to control infection with *V. septique* by antitoxin or antibacterial serum given at a time when sulphapyridine is of little use. *Cl. welchii* antitoxin has a marked therapeutic effect in infection with this strain of the organism but sulphapyridine given only after infection is apparently without action on the course of the disease.

6. In intradermal infection with *V. septique* the combined action of sulphapyridine and antitoxin or of sulphapyridine and antibacterial serum effects a saving in life much greater than would be expected if a mere summation effect was in question. A similar effect was observed in intramuscular infection provided the administration of the drug was sufficiently prolonged. No such synergic effect is produced by the combined action of antitoxic and antibacterial serum.

7. In *Cl. welchii* infection the combined action of antitoxin and sulphapyridine produces a noticeable synergic effect but the evidence on this point is less clearly defined.

8. The pathogenesis of infection with spore-bearing anaerobes is discussed in relation to prophylaxis or therapy with chemotherapeutic agents, antitoxin, antibacterial serum and combinations of such.

9. The statistical aspect of summation effects is briefly considered pending a forthcoming mathematical analysis, by Dr K. Mather, of the figures obtained in experiments on synergic action.

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