BACTERICIDAL SYNERGISM OF NOVOBIOCIN AND TETRACYCLINE AGAINST *PSEUDOMONAS PSEUDOMALLEI*

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MELIOIDOSIS is a severe infectious disease that is endemic in South-east Asia and other regions of the tropics. Clinical manifestations are varied, but the disease is most often recognised as an acute, overwhelming septicaemia with gross and microscopic lesions in the lungs, liver, spleen, lymph-nodes and bone marrow, or as a chronic suppuration which usually affects the lungs but may involve the bones and any other organ. Trauma, stress, malnutrition, chronic intoxications and debilitating diseases are believed to be predisposing factors (Fournier and Chambon, 1958; Rowlands and Curtis, 1965; Magee *et al.*, 1967; Bromwich and Hargrave, 1969; Morrison, 1970; Tarlow, 1971). The causative organism, *Pseudomonas pseudomallei* (Whitmore's bacillus), is found in stagnant water, rice fields, soil and mud-banks in Vietnam (Chambon, 1955a). Contamination of wounds and abrasions, and the inhalation of dust are likely methods of infection for man (Fournier and Chambon).

Antimicrobial therapy presents a difficult problem because of the resistance of *P. pseudomallei* to many of the usual agents, the toxicity of the recommended drugs, the rapid emergence of resistant strains (Fournier and Chambon; Duong-Hong-Mo and Tran-Hiep-Cuong, 1967), the occurrence of remissions (McDowell and Varney, 1947; Alain, Saint-Etienne and Reynes, 1949; Caubet, 1953; Prevatt and Hunt, 1957; Guillermand *et al.*, 1964; Maegraith and Leithead, 1964), and the extreme variation in the course and severity of the disease in different persons (Stanton and Fletcher, 1932; Alain *et al.*). Chloramphenicol, kanamycin, novobiocin and tetracycline are the drugs most commonly used and their simultaneous administration is almost invariably recommended. However, their dosage is a subject of controversy; though some authors claim that only massive chemotherapy can save seriously ill patients (Cooper, 1967; Weber *et al.*, 1969; Thin *et al.*, 1970), others find little justification for the drug doses and combinations that are recommended (Spotnitz, Rudnitsky and Rambaud, 1967; Flemma *et al.*, 1969; Byrd and Puritz, 1970).

There are several reports on the in-vitro sensitivity of *P. pseudomallei* to antibacterial drugs, tested singly (Green and Mankikar, 1949; Brygoo, 1953; Fournier and Chambon; Prevatt and Hunt; Borchardt, Stansifer and Albano, 1966; Patterson, Darling and Blumenthal, 1967; Brundage, Thuss and Walden, 1968; Green and Tuffnell, 1968; Salisbury and Likos, 1970; Fisher, Hillegas and Nazeeri, 1971) or in combination (Chambon, de Lajudie and Fournier, 1954; Chambon, 1955b; Fournier and Chambon; Eickhoff *et al.*, 1970); with few exceptions, the tests used have measured bacteristatic effects only. Clinical experience with melioidosis, as with other serious infections, suggests that the bactericidal effect of drugs in combination may have greater therapeutic relevance.

This report presents the results of a quantitative study to determine the

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sensitivity of six strains of this species to four antimicrobial agents that are usually recommended in the treatment of melioidosis, and their susceptibility to the lethal effects of these agents in combination; detailed consideration of bacteristatic effects was not within the scope of this paper. Analysis of data for evidence of synergism, antagonism or additive effect was made, and is displayed in the form of isobolograms, prepared by a simplification of Loewe's method (1953). A short mathematical presentation of this method is included. Assays were also conducted with Sulfamylon (para-aminomethylbenzene-sulphonamide hydrochloride), a topical antibacterial agent used for the prophylaxis of *P. aeruginosa* infection in burned patients (Lindberg *et al.*, 1965; Moncrief *et al.*, 1966), because of the occurrence of fulminating *P. pseudomallei* septicaemia in American soldiers as a complication of burns and battle wounds (Flemma *et al.*; Foley *et al.*, 1969; Greenawald, Nash and Foley, 1969; Zimmerman, 1970).

MATERIALS AND METHODS

P. pseudomallei strains. The six strains used in this study were obtained from Dr A. D. Alexander, Division of Veterinary Medicine, Walter Reed Army Institute of Research. All had been isolated from American soldiers who had acquired melioidosis in Vietnam, most of them recently. The identity of each strain was confirmed at the Walter Reed Army Institute of Research by the cultural and morphological characteristics of this species and by the appropriate biochemical and serological investigations (Haynes and Burkholder, 1957; Cowan and Steel, 1965; Kishimoto *et al.*, 1971).

The organisms were picked from individual colonies on Brain-heart Infusion Agar (Difco) containing 3 per cent. glycerol and transferred overnight once or twice in Mueller-Hinton (M-H) Broth (Difco) before inoculation into dilutions of antimicrobial agents. *Escherichia coli* strain 80, kindly provided by the Department of Bacteriology, Walter Reed Army Institute of Research, was included in the assays for comparative purposes and tested by the same techniques.

Antimicrobial agents. Four reference standard preparations, supplied by the division of Antibiotics, Food and Drug Administration, U.S. Department of Health, Education, and Welfare, Washington, D.C., were used: chloramphenicol base (1000 µg per mg), kanamycin sulphate (750 µg per mg), sodium novobiocin (850 µg per mg), and tetracycline (1000 µg per mg). All four drugs were weighed out in appropriate amounts to yield the equivalent of 1000 µg per ml stock solution in sterile distilled water. Chloramphenicol was first dissolved in absolute alcohol, and tetracycline in a few drops of 0.1N-HCl; both were further diluted to final concentration with water. All stock solutions were kept refrigerated at 4°C for periods up to 10 days with no loss in potency. Lower concentrations in M-H Broth (200 µg per ml and 50 µg per ml) were prepared on the day of the experiment. Sulfamylon was dissolved in sterile distilled water (200 mg per ml) and assayed in concentrations appropriate for topical use. All chemotherapeutic agents, alone and in combination, were tested in M-H Broth (10 ml total volume), and the final concentration was expressed as µg or mg of drug or drugs per ml.

Drug sensitivity. The sensitivity of the strains to individual drugs was based on the results obtained from tube-dilution tests, with subculture to plates. For each strain, 0.1 ml of an overnight culture at 37°C in M-H Broth was added to tubes of the same broth containing appropriate concentrations of each drug. Controls (growth in broth with no drug) were set up in parallel. An overnight culture contained about 10⁹ bacteria per ml so that initially there were approximately 10⁷ organisms per ml in all tubes. These were incubated for 24 hr at 37°C on a reciprocating shaker, and observations on turbidity were made. On inoculation, and after 24 hours' incubation, 0.1-ml amounts from three or four ten-fold dilutions from each broth tube were spread on the surface of duplicate Trypticase

Soy Agar plates (Difco) by means of a sterile L-shaped rod. Plates were incubated 24–48 hr at 37°C. Colony counts were performed with the aid of an electronic counter. Viable counts were calculated as the average of results of at least two plates for each dilution; readings were made from dilutions that gave evenly distributed colonies with the maximum countable number in the range of 600–1000 colonies per plate.

Criteria for "resistance". As a preliminary to studies of the bactericidal action of drugs and drug combinations, the strains were assessed as "sensitive" or "resistant" to single drugs in terms of the bacteristatic effect of chloramphenicol, novobiocin and tetracycline, and of the bactericidal effect of kanamycin. Strains were considered "resistant" if an increase in viable counts of over five-fold occurred in the presence of 25 µg per ml of chloramphenicol or novobiocin, or 5 µg per ml of tetracycline; and if less than 99.99 per cent. bacteria were killed in the presence of 25 µg per ml of kanamycin. With the exception of novobiocin, the concentrations chosen are comparable with the levels attainable in the blood by means of full dosage. These are (µg per ml): novobiocin 50 to over 100; chloramphenicol 25 (50 is considered a toxic level); kanamycin 10–20; and tetracycline 2–4 (see Garrod and O'Grady, 1968).

Demonstration of synergism

Design of experiments. The bactericidal effects of drugs, alone and in combination, were tested in parallel in broth, with the same inocula, tube-dilution and plating techniques as were described above. The following drug dilutions were used: (a) for single drugs: graded concentrations up to values well above blood levels so as to obtain more than a 95 per cent. bactericidal effect; (b) for drug pairs: concentrations attainable in the blood by full doses or less. These were tested at dose ratios of 1:1, 5:1, 1:5 and 1:25, and, in some instances, at the same ratio but at different absolute concentrations. Combinations were chosen on the basis of the "resistance" pattern of the strains, and doses and dose ratios on the basis of the degree of "sensitivity" to each compound. Growth controls (broth with no drug) were included in each test. After 24 hours' incubation, serial ten-fold dilutions of all tubes were made and 0.1-ml samples at the appropriate dilution were plated. In addition, samples from tubes showing no growth were plated undiluted on to blood agar; this allowed estimates as low as 10 bacteria per ml to be made. Colony counts were recorded as a percentage of the pre-incubation counts.

Mathematical analysis. In this paper, the relationship between dose and response is based on the classical concept of additivity of drugs, according to the following model, a more detailed analysis of which will be published elsewhere. It is assumed that, in the absence of drugs, bacteria grow exponentially; whereas, in the presence of bactericidal drugs, alone or in combination, bacteria are similarly killed exponentially at a rate proportional to the concentration of drug or drugs used. The log response, q , to a drug dose is defined as $\log_{10} \frac{N_0}{N_1}$, where N_0 denotes the number of bacteria present initially (time t_0), and N_1 the bacterial count after a fixed time interval, t_1 (= 24 hr in this study). According to this mathematical model, the log response will vary linearly with the total amount of the drug or drugs tested, so that

$$q = c'z - k' \quad (1)$$

where c' is a constant which denotes the specific bactericidal activity of drugs, single or mixed in fixed proportion, z denotes the amount of drug or drugs, and k' is a constant which denotes the growth rate. In equation (1), k' depends on the strain of bacteria used, while c' depends on both the strain and the drug or drugs tested, and in a single experiment both are constant. In this way, the rate of growth of an organism and the rate of killing due to a drug or drugs are represented on a continuous scale. Negative values of q signify a predominance of growth, and positive values indicate lethal effects; a zero value represents bacteristasis. Examples of this relationship are presented in graphical form in fig. 1.

When two drugs, D_1 and D_2 , having bactericidal activities c'_1 and c'_2 , are tested in combination in the concentrations z_1 and z_2 respectively, the action is said to be additive when

$$q = c'_1z_1 + c'_2z_2 - k' \quad (2)$$

FIG. 1.—The log response (q)* of *P. pseudomallei* strain 6 to four antibiotics tested singly at the concentrations indicated. (○) q values for kanamycin; (+) for tetracycline; (▲) for novobiocin; (●) for chloramphenicol, after 24 hours' incubation. The q value of the drug-free control culture is indicated by the arrow; the horizontal solid line at $q = 0$ corresponds to a bacteriostatic effect; and the dotted line at $q = 2$ represents a 99 per cent. kill.

* $q = \log_{10}$ viable count of bacteria after 24 hours' incubation expressed as a percentage of the inoculum size.

The values of k' , c'_1 , and c'_2 are determined experimentally by testing in parallel assays the growth rate k' in the absence of drugs, and the bactericidal rates c'_1 and c'_2 , respectively, in the presence of each drug of the pair.

The extent to which the experimental data fit the mathematical model of strict additivity implicit in equation (2), can be measured by the degree in which two conditions are fulfilled (Loewe): (a) the log response to doses of a single drug or pair of drugs combined in fixed proportion has a rectilinear graph {equation (1)}, and (b) the isoboles, or lines of equal biological effect, for two drugs combined in variable proportion are also rectilinear. Experimentally, condition (a) can be verified with reasonable accuracy (fig. 1). Deviations, however, may be found in the case of higher drug concentrations or of drug-resistant strains.

In this study, 99 per cent. bactericidal activity (i.e., 1 per cent. viability, $q = 2$), is considered to be the required level of effectiveness of drugs, alone or mixed in fixed proportion. For clarity the experimental drug levels producing a given effect are referred to as "concentrations"; the values obtained by calculation as corresponding to 99 per cent. bactericidal activity are referred to as "doses" (μg per ml). Such doses are estimated by linear inter- or extrapolation only when at least one of the test levels shows more than 95 per cent. bactericidal effect. This requirement is selected so that possible errors, due to deviations from the straight lines implied in condition (a), remain negligible in estimating the points of the isoboles. It is thus possible to construct an isobole for any of the pairs tested. In comparing the isoboles thus obtained with the straight lines implied in condition (b) we say that a given pair of drugs exhibit isobole additivity (referred to as additivity hereafter) if the corresponding isobole is rectilinear, regardless of whether condition (a) is satisfied or not. If, on the contrary, the isobole is either markedly convex or concave towards the origin, the combined effect is said to be synergistic in the former case and antagonistic in the latter (Loewe).

Evaluation of the results. The 99 per cent. bactericidal dose of a single drug is estimated either graphically (fig. 1) or by inter- or extrapolation from the tabulated values (table I), and the data so obtained are summarised in table II. The corresponding doses for each drug in a given combination are estimated similarly (table III); the corresponding graphs are not presented. About 12 combinations per strain were tested. However, if no concentration of a drug, given singly, produced over 95 per cent. bactericidal effect, no estimate of the 99 per cent. effect was attempted, and therefore no test for synergism is recorded. For drugs combined in a given proportion, a bactericidal effect above 95 per cent. was also required; but if a lower effect was obtained when the drugs were tested in parallel, in the same proportion but in different amounts, the data were plotted on the same curve and tabulated for inter- or extrapolation, and were used in calculating the co-ordinates of the corresponding point in the isobole.

In constructing isobolograms for pairs of drugs (fig. 2), the dose of each drug needed for its independent effect (table II) is measured on the corresponding co-ordinate axis as one unit (arithmetic scale); the weight equivalent of one unit is indicated in table II. The plotted points denote the relative doses of individual drugs (table III) that are mixed in variable proportion, in combinations that evoke the same biological response. The curve fitted to these points is the isobole and the shape of this curve suggests the type of combined action. In the present study, a synergistic effect is assumed to warrant clinical consideration when the concentration of each drug in a pair is 0.2 unit or less; accordingly, the corresponding isobole is markedly convex. The use of a biological unit scale is essential in comparing the relative effect of different pairs and indicates more clearly the upper limit of synergism for a pair of drugs. The same type of relative scale is also used by Hewlett (1969) in presenting various models for non-linear isoboles.

RESULTS

"Resistance" patterns of test strains

From the preliminary tests it was concluded that strains 1, 2, 3, and 6 were "resistant" to chloramphenicol (25 μg per ml), strains 1, 2, 3, 4, and 5

to kanamycin (25 μg per ml), and strains 1, 2, and 3 to tetracycline (5 μg per ml), but that all six strains were "sensitive" to novobiocin (25 μg per ml).

TABLE I

Log response (q) of six strains of Pseudomonas pseudomallei and one strain of Escherichia coli to chloramphenicol, kanamycin, novobiocin or tetracycline in broth cultures

Drug	Concentration (μg per ml)	q^* values for						
		<i>P. pseudomallei</i> strain number						<i>E. coli</i> strain 80
		1	2	3	4	5	6	
Chloramphenicol	1	...	...	-2.03	-1.90	-1.76	...	...
	5	-2.21	-1.97	-1.66	-1.04	-1.25	-1.94	-0.98
	25	-2.05	-1.21	-0.98	0.60	0.21	-1.55	2.49
	100	-1.46	1.77	1.52	3.25	3.00	-0.56	...
Kanamycin	0.2	-2.33	...	...	...	...	-1.83	-2.20
	1	-2.21	...	...	-2.05	-1.57	-0.85	-0.90
	2	...	...	...	...	...	...	1.05
	4	...	...	...	...	...	...	5.04
	5	-1.52	-1.45	-1.62	-1.74	-1.08	1.20	...
	10	...	...	...	...	...	3.21	...
	25	1.42	1.27	-0.49	-0.62	0.30	...	...
Novobiocin	0.2	...	...	...	...	...	-2.05	...
	1	...	...	-2.03	...	-1.53	...	...
	2.5	...	...	...	...	...	-1.75	...
	5	-1.62	-1.48	-1.85	-1.74	-1.01	...	-1.70
	10	...	...	-1.50	-1.40	-0.70	...	...
	25	-0.60	-0.25	-0.33	-0.37	0.20	0.29	1.40
	50	...	...	...	...	...	...	3.45
Tetracycline	100	2.57	2.03	1.92	2.19	2.60	3.54	...
	1	-2.12	...	-2.02	-1.41	-1.39	-1.80	...
	2	...	...	-1.57	-1.16	-1.14	...	...
	2.5	...	...	...	...	...	-1.21	...
	5	-1.10	-0.96	-0.97	-0.50	-0.40	-0.51	-0.35
No drug	25	1.81	2.58	2.03	2.12	2.26	2.66	3.00
	...	-2.37	-2.05	-2.08	-2.14	-1.75	-2.11	-2.31

* $q = \log_{10}$ viable count of bacteria after 24 hours' incubation expressed as a percentage of the inoculum size.

Bactericidal action of drugs tested alone or in combination

Results obtained with single drugs. The effect of several concentrations of the drugs on the number of organisms present after 24 hr is shown in table I. As regards the "sensitivity" or "resistance" of the strains, the results agreed reasonably well with those of the preliminary tests. When analysed graphically, as exemplified in fig. 1, or by calculation, the relationship of the log response, q , of a strain to increasing concentration of a particular drug was essentially

linear. From these data, the doses producing a 99 per cent. bactericidal effect were estimated (table II). Novobiocin showed this degree of activity against

TABLE II

*Doses of single drugs (μg per ml) estimated as producing 99 per cent. bactericidal activity ($q = 2$) against six strains of *P. pseudomallei* and one strain of *E. coli***

Drug	<i>P. pseudomallei</i> strain number						<i>E. coli</i>
	1	2	3	4	5	6	
Chloramphenicol	n.e.	132.5	114.4	64.0	73.0	n.e.	22.1
Kanamycin	29.0	30.5	n.e.	n.e.	n.e.	7.0	2.5
Novobiocin	86.4	99.2	103.0	95.0	82.0	64.4	35.0
Tetracycline	26.2	21.9	25.0	24.0	23.0	21.0	19.0

* Each dose value shown is regarded as one bactericidal unit for the drug acting on the bacterial strain. n.e. = Not suitable for extrapolation.

all six *P. pseudomallei* strains, but at concentrations that can be reached systemically only by means of unusually high dosage (64.4 to 103 μg per ml).

TABLE III

*Log response (q) of five strains of *P. pseudomallei* to the combined action of novobiocin and tetracycline in broth cultures. Estimated and relative doses of each antibiotic for 99 per cent. bactericidal activity of the combination*

Strain number	Drug concentrations tested (μg per ml)	q	Estimated dose* for $q = 2$ (μg per ml)	Relative dose†	Drug effect‡
2	N 25.0, T 5.0	2.66	N 21.5, T 4.30	N 0.22, T 0.20	(S)
	N 5.0, T 5.0	2.46	N 4.50, T 4.50	N 0.04, T 0.20	S
3	N 10.0, T 2.0	2.21	N 9.44, T 1.89	N 0.09, T 0.07	S
	N 5.0, T 5.0	2.03	N 4.92, T 4.92	N 0.05, T 0.19	S
4	N 10.0, T 2.0	2.23	N 9.40, T 1.88	N 0.10, T 0.08	S
	N 5.0, T 5.0	2.21	N 4.72, T 4.72	N 0.05, T 0.20	S
5	N 10.0, T 2.0	2.52	N 8.76, T 1.75	N 0.11, T 0.08	S
	N 5.0, T 5.0	2.37	N 4.54, T 4.54	N 0.06, T 0.20	S
6	N 2.5, T 2.5	2.65	N 2.15, T 2.15	N 0.03, T 0.10	S
	N 0.2, T 5.0	2.55	N 0.18, T 4.40	N 0.003, T 0.20	S

* For "no drug" data required for estimation of these values see table I.

† Dose of each drug expressed as a fraction of one unit of drug acting alone (see table II). N = Novobiocin; T = tetracycline.

‡ S = Marked synergism; (S) = moderate synergism.

Chloramphenicol and tetracycline required concentrations that are above tolerable blood levels, and the same was true of kanamycin for five of the

FIG. 2.—Isobolograms of estimated 99 per cent. bactericidal action of selected drug combinations on four strains of *P. pseudomallei*, at 24 hr. For each drug, 1.0 drug unit corresponds to the estimated dose of the drug which, when it is acting alone, produces a 99 per cent. kill. In each case the dotted line represents a theoretical purely additive effect of two drugs in combination; the pecked lines indicate the maximum tolerated blood concentrations of the corresponding drugs. The bacterial strains and the drug doses (μg per ml) corresponding to 1.0 unit are as follows (drugs indicated by their initial letters): strain 3: N 103, T 26; strain 5: N 82, C 73; strain 4: C 64, T 24; strain 6: *left*, K 7, T 21, and *right* K 7, N 64.

strains. Satisfactory lethal action on the *E. coli* strain by concentrations reached in the blood was confirmed for chloramphenicol, kanamycin and novobiocin.

Results obtained with drug combinations. Six combinations, tested in repeated assays, attained 99 per cent. lethal effect, and the corresponding iso-

FIG. 3.—A comparison of the viable counts of five strains of *P. pseudomallei* after 24 hours' exposure to concentrations of novobiocin and tetracycline alone or in combination. The length of each column represents the number of bacteria as a percentage of the number inoculated. Hollow columns: numbers in cultures without added drug; banded columns: in cultures with novobiocin; hatched columns: with tetracycline; solid columns: with both drugs.

boles are either synergistic or additive. Of these pairs, only the novobiocin-tetracycline combination was highly synergistic; the pertinent data from a typical experiment are presented in table III, and the corresponding isobole for strain 3 is shown in fig. 2 (upper panel). This isobole exemplifies the reactions of strains 2, 3, 4, 5 and 6 which appeared to differ only minutely from each other. The plots were, indeed, markedly convex for these five

strains, of which three were "sensitive" to both drugs (strains 4, 5 and 6) and two to novobiocin only (strains 2 and 3). The effectiveness of the novobiocin-tetracycline combination is readily apparent when the percentage of viable bacteria is plotted in terms of drug pairs and of each drug in the pair (fig. 3). It can be seen that bacteria were killed in the presence of both drugs at concentrations in which they allowed growth when used alone (2.5 to 10 μg per ml of novobiocin and 2 to 5 μg per ml of tetracycline). This bactericidal synergistic effect (0.2 per cent. to 0.6 per cent. survival) was demonstrable with concentrations as low as 0.2 μg per ml of novobiocin in the combination (table III, strain 6), and was unaffected by the pattern of resistance to the other drugs.

The patterns of response to the other combinations varied, and the detailed data are omitted from table III. Generally, a moderate synergism or an addi-

TABLE IV

Effect of exposure to Sulfamylon for 24 hr on the viability of P. pseudomallei in broth cultures

Strain number	Log ₁₀ number of viable bacteria per ml after contact with				
	no drug	Sulfamylon (mg per ml)			
		5	10	50	100
1	8.7	7.9	6.5	6.1	<1*
2	9.2	8.6	6.6	6.1	4.0
3	8.6	8.0	6.4	4.8	<1
4	8.6	8.3	6.3	5.2	<1
5	9.0	8.4	6.5	5.9	2.2
6	8.8	8.6	6.3	5.6	2.4

* No growth on plate subcultures.

tive effect was suggested; the other graphs in fig. 2 show isoboles that exemplify these reactions. For moderate synergism, however, fairly high concentrations of chloramphenicol, kanamycin or tetracycline were needed in the combinations chloramphenicol-novobiocin (strains 3, 4 and 5), chloramphenicol-tetracycline (3 and 4), kanamycin-tetracycline (strain 6 and *E. coli*), kanamycin-novobiocin (strain 6), and kanamycin-chloramphenicol (*E. coli*). Thus, for some strains, the concentration of chloramphenicol was close to or within the toxic range and that of tetracycline was near to or somewhat above the upper limit of the blood level (fig. 2, strains 4, 5 and 6). Except in the case of strain 6, the degree of kanamycin resistance was too high to permit study of this drug in combination. With this strain, the kanamycin concentration required was as much as half that needed for its independent effect; see fig. 2 (lower panels). Chloramphenicol combined with novobiocin or tetracycline, and kanamycin with either chloramphenicol, novobiocin or tetracycline had little or no bactericidal effect on any other strain for which a test of synergism was possible. No combination was bactericidal to strain 1.

Data for the *E. coli* strain are not shown in table III. The novobiocin-

tetracycline combination had no lethal effect on this strain; other combinations were highly lethal but not highly synergistic.

The bactericidal effect of Sulfamylon

Sulfamylon, when tested at a concentration (100 mg per ml) appropriate to its topical use in a 10 per cent. cream (Lindberg *et al.*) was totally bactericidal for three of the six strains of *P. pseudomallei*, and highly so to the remainder (0.001, 0.003 and 0.08 per cent. survival respectively: table IV).

DISCUSSION

This report presents, to our knowledge, the first detailed quantitative study of the bactericidal activity against *P. pseudomallei* of combinations of drugs used in the treatment of melioidosis. The earlier observations of Chambon and co-workers (Chambon *et al.*, 1954; Fournier and Chambon, 1958) should be interpreted with caution because of the techniques and the criteria adopted. They reported as evidence of bactericidal synergism any decrease in colony counts as compared with those from the inoculum; however, such findings could include effects which are no more than additive. Moreover, the small inocula they used were unlikely to reveal resistant variants. Our studies showed that all strains were sensitive to novobiocin. This observation is in agreement with the findings of Vietnamese (Duong-Hong-Mo and Tran-Hiep-Cuong, 1967) and American investigators (Brundage *et al.*, 1968), who noted a uniform behaviour of *P. pseudomallei* toward this antibiotic. Moreover, it was found in this study that novobiocin alone is bactericidal at concentrations that can be reached systemically only by unusually high dosage, whereas when it was combined with tetracycline a bactericidal synergistic effect was obtained with as little as 0.2 μ g per ml, or 0.003 of the amount needed for a comparable independent effect. This is good evidence of the potency of this combination *in vitro*; though its efficacy remains to be demonstrated in controlled clinical trials, this synergistic mixture may be clinically effective, and simultaneously provide a means of thwarting the emergence of resistant strains.

Chloramphenicol-resistant strains have been reported by Caubet (1953) and many other workers (Chambon *et al.*; Chambon, 1955*b*; Fournier and Chambon; Duong-Hong-Mo and Tran-Hiep-Cuong). Resistance to chloramphenicol, to tetracycline, and to both in combination have been found to occur during the course of treatment (Chambon, 1955*b*; Chambon *et al.*). In agreement with these findings, we observed the development of chloramphenicol resistance in one patient (strain 6; Calabi, unpublished data), and resistance to both chloramphenicol and tetracycline in three of the four chloramphenicol-resistant strains. Because our *in-vitro* data suggest that the chloramphenicol-tetracycline combination is either additive or only moderately synergistic, this type of combined therapy does not appear to show much promise. The same may be said of the chloramphenicol-novobiocin combination and the combinations of kanamycin with tetracycline or novobiocin.

The topical use of Sulfamylon in wounds and extensive burns was first

advocated in World War II for the prevention of gas gangrene (Garrod and O'Grady, 1968). Since 1964 this compound has proved to be of great benefit in the management of burn sepsis caused by *P. aeruginosa* (Lindberg *et al.*, 1965; Moncrief *et al.*, 1966), *Proteus*, *Staphylococcus aureus* and other organisms (Monafo, 1967, cited by Garrod and O'Grady, p. 300). The in-vitro studies recorded here showed that Sulfamylon was completely bactericidal against some *P. pseudomallei* strains and highly bactericidal against the rest. These findings suggest that prompt topical application of the drug to burns and battle wounds might reduce the frequency of melioidosis.

The mathematical model of strict additivity (or linearity) of drug action has long been known, and has been applied by Loewe (1953) and many other workers in biological systems. Deviations from the linear model exist, and several models have been proposed in order to predict or correlate such deviations (Hewlett, 1969). This study uses Loewe's method of isobolograms in a simplified form that appears adequate for our purposes. The results showed that most of the combinations tested deviated from strict additivity (equation (2)) since the isoboles for 99 per cent. bactericidal activity usually indicated a marked or a moderate synergistic effect. However, additivity of drugs is consistent with the experimental data in the case of some strains when rather large doses of chloramphenicol were tested in combination with tetracycline or novobiocin, as shown in fig. 2 (strains 4 and 5) respectively. Moreover, additivity cannot be excluded in the case of the combination of kanamycin with tetracycline or with chloramphenicol against the *E. coli* strain (not illustrated). On the other hand, the property of linearity implicit in equation (1) was verified to a reasonable degree with all strains. This was true for most drugs tested singly, particularly at the lower concentrations, and also in the case of drugs combined in a fixed proportion but at different concentrations.

SUMMARY

On the assumption that bactericidal action of drugs is probably required for the effective treatment of melioidosis, in-vitro experiments were performed with five chemotherapeutic agents and six *Pseudomonas pseudomallei* strains from clinical sources. Chloramphenicol, kanamycin, novobiocin and tetracycline, alone and in various combinations, were tested for bactericidal activity against reasonably heavy inocula by means of a tube-dilution technique with subculture for viable counts. Results are displayed by Loewe's method of isobolograms in a simplified form. The novobiocin-tetracycline combination produced a kill of 99.4 to 99.8 per cent. with five of the six strains at concentrations at which neither drug had any effect when used alone (2.5 to 10 μg per ml of novobiocin and 2 to 5 μg per ml of tetracycline). The synergistic effect of this mixture was demonstrable with as little as 0.2 μg per ml of novobiocin, or 0.003 of the concentration needed for its comparable independent effect; in clinical use the latter could be achieved systemically only with unusually high dosage. No bactericidal effect was found with one strain.

Sulphamylon showed a strong bactericidal effect at concentrations that might be achieved locally by topical applications to wounds and burns.

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