Methods of testing combined antibiotic bactericidal action and the significance of the results

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SYNOPSIS A description is given of two methods of measuring combined antibiotic bactericidal action: a test in liquid medium with subculture and the cellophane transfer method.

It is emphasized that information so obtained is necessary in order to predict the effect of combined treatment, particularly in bacterial endocarditis due to organisms not fully sensitive to penicillin. Eight case histories are given, in all of which such a prediction was fulfilled, one of failure and seven of success from the use of five different combinations.

The cellophane transfer method was applied to the study of the nature of combined antibiotic action on multiple strains of several bacterial species. The results were rarely uniform for any given combination and species: the necessity for individual tests as a guide to treatment is thus confirmed.

Modifications of the theory of combined action formulated by Jawetz are proposed.

The advisability of prescribing antibiotics in combinations rather than singly is a long-standing subject of controversy. There are five usually accepted indications for combined treatment (Garrod, 1953), one of which is the attainment of a synergic effect. When the indication is of a different nature, such as an attempt to prevent acquired bacterial resistance, it is advisable to remember that a given combination may be either synergic, merely additive, or actually antagonistic, in accordance with the law formulated by Jawetz and Gunnison (1952). Although there are many individual exceptions to this, it is generally true that synergy is rarely seen except in the action of a pair of bactericidal drugs, and that antagonism only results from combining a bactericidal with a bacteriostatic. The reality of these effects, not only *in vitro* but in the experimental animal and in the clinical field itself, is indisputable.

Jawetz and others have emphasized that the action of a combination on a given organism cannot be certainly predicted on theoretical grounds. It requires verification, because not all strains behave alike. It is with methods for doing this that this paper is concerned. They are called for only in connexion with the treatment of seriously ill patients, notably those with bacterial endocarditis. The object is then to identify a combination which is completely bactericidal for the infecting organism, assuming

that penicillin alone has been found not to exert this effect. It cannot be emphasized too strongly that the object of treatment in this disease should be to sterilize the lesion: antibiotics which merely inhibit bacterial growth arrest its progress for as long as they are given but cannot eradicate the infection. For this reason tests of combined action depending merely on inhibition of growth are inapplicable and will not be considered here. Tests of bactericidal action are of the following two kinds.

TESTS IN LIQUID MEDIA WITH SUBCULTURE

If this test is to embrace a number of antibiotics, and unless it is to be inordinately complicated, each can be used in only a single concentration, which should be one attainable in the blood by full doses. In the method devised by Martin, Sureau, and Chabbert (1952), who adduce abundant clinical evidence of the significance of their results, antibiotics are added to broth in a fixed concentration, singly and in every possible combination, these tubes being inoculated with the organism and plated after overnight incubation. The most promising combination is that giving the fewest survivors. The technique of the test is described in more detail by Chabbert (1953a and b) who describes both an elaboration of it using multiple antibiotic concentrations, and two means of simplifying it: these are adding the antibiotics on discs which are prepared in quantity and stored, and pre-inoculating the medium used in

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bulk. A somewhat more elaborate form of this test is described by Jawetz, Gunnison, Coleman, and Kempe (1955), which begins by testing the inhibitory action of two concentrations of each antibiotic, the lower of these being used in combined tests if it proves capable of inhibiting growth.

TRANSFER METHODS

REPLICA PLATE In this method, derived from that of Lederberg and Lederberg (1952), as described by Elek, Hilson, and Jewell (1953), Elek and Hilson (1954), and Manten (1954, 1956), antibiotic-containing discs are placed on a heavily inoculated plate, producing a zone of inhibition. A replica plate, inoculated by transfer on a velvet pad, indicates whether this zone contains survivors. Each antibiotic should also be tested singly: combined action is determined either by placing the two different discs near together or by including both antibiotics in one disc. From the results obtained it is evident that this method gives significant results: the objection to it is that velvet transfer carries over only about 1% of the bacteria on the primary plate. What appears to be complete bactericidal action may therefore not be: indeed, it is not possible by this method to determine that an antibiotic or combination has sterilized the inoculum, and the object of such a test is to identify a combination which will do this and no less.

CELLOPHANE TRANSFER The foregoing objection does not apply to this method, since the whole operation is conducted on one surface, and every survivor from the period during which the antibiotics were acting can form a colony. Details of this elegant and revealing method devised by Chabbert (1957, 1960) are given in the succeeding section. In outline, it consists of applying blotting paper strips containing different antibiotics as a rectangle on a plate of medium, and allowing time for their diffusion before removal. A cellophane 'tambour' is then applied, the interior surface of which has been heavily inoculated with the organism. Both nutrients and antibiotics diffuse through the tambour. After preliminary incubation on the original plate, the tambour is transferred to a plate of normal medium, and bacteria surviving in the zone of inhibition will then form colonies.

The final picture shows at a glance to what extent each antibiotic is bactericidal when acting alone, and, from what happens in the area surrounding the angle where the strips meet, how they act in combination. This area may be the only part of the plate free of colonies (synergy) or may be occupied by a band of profuse growth while alongside the strips

more remote from the angle colonies are few (antagonism). Between these extremes are other appearances indicating lesser degrees of both types of combined action. A notable advantage of the method is that in the critical area of the culture the two separate antibiotic diffusion gradients produce a range of relative concentrations, and it may be evident from the result that a certain effect is obtained only, or is more marked, when the concentration of one antibiotic exceeds that of the other. According to Chabbert and Patte (1960), such findings can successfully be translated in terms of therapeutic dosage.

We have employed each of these principal methods, the first almost exclusively as a guide to the treatment of individual patients, the second (cellophane transfer) also for this purpose but with another object in addition. This was to obtain a general picture of the nature of combined action among the more commonly used antibiotics. For this purpose it was necessary to test multiple strains of some of the 'problem' bacteria. It was hoped also to determine whether the cellophane transfer method gives more consistent results than others.

METHODS

LIQUID MEDIUM WITH SUBCULTURE Antibiotics are dissolved in broth in a concentration of 100 µg./ml. (penicillin and bacitracin respectively 100 and 2.5 units/ml.) and 0.5 ml. volumes are pipetted into tubes, singly and in every possible combination. These tubes may conveniently be accommodated in a square rack, arranged as shown in Fig. 1, an arrangement which we are accustomed to refer to as the 'half chess-board'. The number of tubes required varies from 15 for a test employing five antibiotics to 55 for one employing 10. To each of these tubes is then added 4.5 or 4 ml. (according to whether they contain one or two antibiotics) of broth preinoculated with the organism to be tested. The medium requires no addition if the organism is a staphylococcus or *Str. faecalis*: for other streptococci the addition of 5% of blood is advisable. The inoculum should be a reasonably heavy one: we have added 2 drops of undiluted broth culture to 100 ml. of broth, which results in a viable content of $>10^5$ /ml. One loopful from a control tube containing no antibiotic is plated to give a standard with which the bacterial content of the other tubes can later be compared.

After overnight incubation a full 2 mm. loopful from each tube showing no growth is plated on blood agar. Plates of ordinary size may conveniently be divided into quarters for this purpose, but care must be taken to distribute the inoculum as widely and uniformly as possible: if it is concentrated in a small area, carried-over antibiotic may inhibit growth. If the antibiotic is purely bacteristatic, the number of colonies is of the same order as in the control culture (denoted by '+' in Fig. 1). Bactericidal action may be partial, the numbers of

colonies being diminished, sometimes to a very small number, or complete, the subculture being sterile ('+' and '-' respectively).

CELLOPHANE TRANSFER METHOD

CELLOPHANE TRANSFER METHOD Strips of sterilized Ford blotting paper 0.5×5 cm. are immersed completely in antibiotic solutions, briefly drained of surplus solution by application to dry blotting paper, and placed at a right angle to one another with the angles in contact on the surface of a blood agar plate: this is incubated overnight to permit diffusion. This use of freshly impregnated strips is a convenient modification, when large numbers of tests are not being done, of Chabbert's method of preparing dried strips which are stored. The strengths of antibiotic solutions used are penicillin G 600 $\mu\text{g./ml.}$, methicillin, streptomycin, neomycin, kanamycin, polymyxin, and vancomycin, 1,000 $\mu\text{g./ml.}$, tetracycline, chloramphenicol, novobiocin, and erythromycin 200 $\mu\text{g./ml.}$ These are the amounts used by Chabbert, but it is arguable that for some purposes some of them are too high. For testing a highly sensitive organism the concentration of penicillin is better reduced: this was done in the test of a pneumococcus shown in Fig. 3F.

The tambour is prepared by stretching a circle of cellophane (those sold as jam pot covers are suitable) over a glass cylinder 7.5 cm. in diameter and 2.5 cm. deep, on which it is held in place by two rubber bands, the whole being sterilized by autoclaving. The inner surface of this is inoculated by flooding with a 1 in 10 dilution of a broth culture of the organism, the surplus being removed from the tilted tambour with a Pasteur pipette. The tambour is then placed on the surface of the plate from which the antibiotic strips have been removed, covered with a filter paper and transferred to the incubator. When the surface of the tambour is dry, the whole is inverted. After seven hours' incubation the tambour is removed from the original plate and transferred to a second plate of medium containing no antibiotics and incubated overnight. Blood agar is the usual medium, but others may be used, e.g., 'chocolate' agar for *H. influenzae* or simple nutrient agar with no addition for hardier species. The surface of the second plate should be dry before the tambour is applied.

RESULTS

TESTS IN LIQUID MEDIUM WITH SUBCULTURE We employed some variant of this method for over 10 years: the results with penicillin-resistant streptococci from cases of endocarditis (including three strains of *Str. faecalis*) are referred to by Cates, Christie, and Garrod (1951). The usual synergic action of penicillin and streptomycin on *Str. faecalis* has been confirmed repeatedly since that time.

More recently a fuller range of tests of combined action has been carried out, as described in the preceding section, on bacteria from patients with endocarditis not only in this hospital, but in others,

whence the culture has been referred for examination. In some instances penicillin alone proved to be fully bactericidal, and treatment with it was successful. In others the disease was far advanced, and death from heart failure occurred at too early a stage for the results of treatment to be assessed. The patients whose brief case histories follow were of more interest. The first was a predicted failure in treatment: in the remainder several different combinations were recommended and treatment with them was successful. Their combination antibiograms are given in Fig. 1.

Case 1 A.W., a man aged 37, developed an endocarditis of the aortic valve due to *Str. faecalis* which was diagnosed in a provincial hospital in February 1957. He had had teeth extracted four months before, and 'cystitis' (with pain in the loin and 'brown' urine) two months before: in view of the nature of the infection the latter episode seems the more likely origin of the infection. He was treated from February until the end of May, first with penicillin + streptomycin + Benemid, then with streptomycin + erythromycin, but the fever remained uncontrolled. He was transferred to the National Heart Hospital, whence we received his culture, on May 29 under the care of Dr. Paul Wood. This organism proved to be abnormally resistant to penicillin (M.I.C. 6 $\mu\text{g./ml.}$ by tube dilution), and, although it was normally sensitive to streptomycin (10 $\mu\text{g./ml.}$), this combination did not kill it nor did any other in a 55-tube test. It was not possible to suggest any combination likely to succeed. Seven different antibiotics, some in combinations, were given without effect, and after progressive deterioration, the patient died on July 7. At necropsy all three cusps of the aortic valve were found to be affected, and there was an aneurysm of the sinus of Valsalva, which had ruptured shortly before death.

Case 2 W.S., a man aged 47, was admitted to St. Bartholomew's Hospital in February 1958 with an endocarditis due to *Str. faecalis* apparently precipitated by dilatation of a urethral stricture. This organism was inhibited by 4 units (2.4 $\mu\text{g.}$) penicillin per ml., but only by 50,000 $\mu\text{g./ml.}$ of streptomycin, and this combination, as might be expected from the high degree of resistance to streptomycin, was not totally bactericidal, nor was any other in a 36-tube test found to have such an effect (see Fig. 1). A course of penicillin alone was followed by relapse, and it was then found that the combination penicillin + neomycin was totally bactericidal: a course of treatment with these two antibiotics purchased full recovery at the price of total loss of hearing. This case is fully described by Havard, Garrod, and Waterworth (1959).

This result may be compared with that in a patient described in the following section, treated for six weeks with penicillin + kanamycin and recovering without any eighth nerve damage.

Case 3 R.K.G., a man aged 27, with a history of rheumatic fever 12 years earlier, was admitted to Queen Mary's Hospital, Roehampton, under the care of Dr. G.

	Penicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin	Vancomycin	Bacitracin	Oleandomycin	E129 (ostreogricin)
Penicillin	+	+	+	+	+	+	+	+	+	+
Streptomycin		+	+	+	(+)	+	+	(+)	+	(+)
Tetracycline			+	+	+	+	+	+	+	+
Chloramphenicol				+	+	+	+	+	+	+
Erythromycin				+	+	+	+	+	+	+
Novobiocin					+	+	+	+	+	+
Vancomycin						+	+	+	+	(+)
Bacitracin							+	+	+	+
Oleandomycin								+	+	+
E129 (ostreogricin)										+

Case 1

	Penicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin	Vancomycin	Bacitracin
Penicillin	+	+	+	+	(+)	(+)	+	+
Streptomycin		++	++	+	+	++	+	++
Tetracycline			++	+	+	++	+	++
Chloramphenicol				+	+	+	+	+
Erythromycin					+	+	+	+
Novobiocin						++	+	++
Vancomycin							+	+
Bacitracin								++

Case 2

	Penicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin	Vancomycin	Bacitracin
Penicillin	(+)	-	+	(+)	(+)	-	(+)	-
Streptomycin		-	(+)	+	(+)	(+)	-	+
Tetracycline			+	+	+	+	+	+
Chloramphenicol				+	+	+	(+)	+
Erythromycin					+	+	(+)	(+)
Novobiocin						+	(+)	+
Vancomycin							(+)	(+)
Bacitracin								++

Case 3

	Penicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin	Vancomycin	Ristocetin	Neomycin	E129 (ostreogricin)
Penicillin	(+)	-	-	(+)	-	-	(+)	-	-	-
Streptomycin		++	-	(+)	-	(+)	-	-	++	-
Tetracycline			(+)	-	-	-	-	-	-	-
Chloramphenicol				(+)	-	(+)	(+)	(+)	-	-
Erythromycin				-	-	-	-	-	-	-
Novobiocin					-	(+)	(+)	(+)	(+)	-
Vancomycin						(+)	-	-	-	-
Ristocetin							-	-	-	-
Neomycin								++	++	-
E129 (ostreogricin)										-

Case 4

	Penicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin	Vancomycin	Ristocetin	Neomycin	Bacitracin
Penicillin	+	+	+	+	(+)	(+)	-	+	-	-
Streptomycin		(+)	(+)	(+)	(+)	-	-	(+)	-	-
Tetracycline			(+)	(+)	(+)	(+)	(+)	(+)	-	(+)
Chloramphenicol				+	(+)	(+)	(+)	(+)	-	-
Erythromycin					(+)	(+)	(+)	(+)	-	(+)
Novobiocin						-	-	(+)	-	-
Vancomycin							(+)	-	-	(+)
Ristocetin								+	-	-
Neomycin									-	-
Bacitracin										+

Case 5

FIG. 1. Combination antibiograms for Cases 1 to 5.

++ = growth

(+) = partial bactericidal action

+ = bacteristasis (numbers as in control)

- = complete bactericidal action

Hosking, on 5 November 1957, having been febrile for several weeks. He had recently had teeth extracted. He had signs of both mitral and aortic lesions, and blood culture yielded *Str. viridans*. This organism required 0.5 unit/ml. (0.3 µg.) of penicillin to inhibit its growth, and penicillin was not totally bactericidal, but penicillin + streptomycin was (Fig. 1). He was treated with penicillin alone from 6 November and from 9 November with penicillin + erythromycin, but in view of these findings he was given from 22 November penicillin 1.5 mega unit four-hourly (later reinforced with benemid) and 0.5 g. streptomycin b.d. This treatment was continued with short intermissions for two months, despite some febrile episodes, one of which was associated with a severe *Ps. pyocyanea* urinary tract infection. He recovered, and is still well and active four years later.

Case 4 A full history of D.D., a man aged 40, has been given by Garrod and Waterworth (1962). Briefly, he was found to have an endocarditis due to a *Str. viridans* normally sensitive to penicillin and other antibiotics, and had a full course of treatment with penicillin and streptomycin. Fever recurred soon after this was completed, and blood culture was again positive, the streptococcus being now highly penicillin-resistant. The first inquiry made by one of us (L.P.G.) was whether any teeth had been extracted during the course: this proved to be so, multiple extractions having been done three weeks after the course started. The streptococcus was abnormally resistant only to the two antibiotics administered (M.I.C. penicillin 8 µg./ml.: streptomycin > 512 µg./ml.) and a selective effect of treatment on the oral flora was evident, with extractions resulting in re-infection with a streptococcus having these unusual characters. Despite its high resistance to these two antibiotics, the combination antibiogram (Fig. 1) was highly encouraging: even the combination penicillin + streptomycin was totally bactericidal. That recommended was penicillin + erythromycin, both because of greater ease and safety of administration and because erythromycin was totally bactericidal alone and in every combination. This treatment was given and was successful.

Case 5 J.S., a girl aged 3 years, and known to have a patent ductus arteriosus, became ill with high fever while resident in Ibadan in June 1958. After unsuccessful treatment at home with penicillin, tetracycline, and chlortetracycline she was admitted to University College Hospital, Ibadan, where a penicillin-resistant *Staph. pyogenes* was grown from her blood. Treatment successively with chlortetracycline and erythromycin, to both of which the organism was sensitive, had little effect, and she was transferred to the London Hospital under the care of Dr. Wallace Bridgen. Her temperature, on erythromycin, was 99 to 101°F.: when treatment was stopped to obtain a further culture it rose to 105°F. This culture (obtained on 24 August) kindly referred to us by Professor C. F. Barwell, was sterilized by several combinations (see Fig. 1): those including vancomycin, neomycin, and bacitracin were naturally not favoured, and the most promising appeared to be streptomycin and novobiocin, particularly since the latter was bactericidal even when acting alone. As from 29 August, the child was accordingly given streptomycin 0.2 g. intramuscularly b.d. and novobiocin

0.25 g. q.d.s. orally. The temperature fell to normal in 48 hours and remained so for about 10 days, when it began to rise again (? cause). The ductus was ligated by Mr. G. Flavell on 15 September and the child made an uninterrupted recovery.

Case 6 C.G., a girl aged 10 with a ventricular septal defect (Roger type), developed endocarditis in December 1958. *Str. faecalis* was cultivated from her blood. She was treated elsewhere for one month with penicillin and tetracycline, for two months with tetracycline only, and for three months with erythromycin, after which she was admitted to the Hospital for Sick Children, Great Ormond Street, under the care of Dr. R. E. Bonham Carter on 24 June 1959. Our initial investigations in this case were confined to showing that the combination of penicillin + streptomycin was totally bactericidal for this strain of streptococcus, and she was treated with these two antibiotics for three months, making a complete recovery. She had 0.5 g. streptomycin b.d. throughout: initially 5 mega units penicillin were given six-hourly, but after two weeks of this the hope was expressed that a régime could be devised involving less frequent injections. It was suggested that the two daily injections should contain penicillin 2 mega units with streptomycin 0.5 g. and that six hours after each, penicillin V 2 mega units and benemid 0.5 g. be given orally; blood assays showed that this treatment maintained adequate levels of both antibiotics.

This case illustrates three points: the inefficacy of the 'static' antibiotics in this disease, treatment for no less than six months having failed, the efficacy of the classical combination, and the possibility of making its administration more tolerable for a child who had had a very rough time.

CELLOPHANE TRANSFER METHOD Typical results are illustrated in Figs. 2 and 3. They are of three kinds.

Indifference The zones of effect (Fig. 2A) form a right angle, scarcely blunted at all. Streptomycin is completely bactericidal and vancomycin incompletely so, but there is no extension of the streptomycin effect where this antibiotic is diffusing into the other zone.

Synergy Fig. 2B shows synergic action by vancomycin and kanamycin against *Str. faecalis*. (This formidable combination of two ototoxic drugs is not likely to commend itself therapeutically except as a last resort.) There are many survivors in the remoter part of the vancomycin zone, but the effect of kanamycin extends deeply into this, converting it into total bactericidal effect where the kanamycin concentration must be quite low. Fig. 2C is an example of the most frequently observed synergic effect, that of penicillin and streptomycin on *Str. faecalis*. The penicillin (vertical) zone shows the paradoxical zone phenomenon (Eagle and Musselman, 1948), bactericidal action being more nearly complete in a band

FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

FIG. 2E

FIG. 2F

FIG. 2. Results of tests by the cellophane transfer method.

Organism	Antibiotic	
	Vertical	Horizontal
A <i>Staph. pyogenes</i>	Vancomycin	Streptomycin
B <i>Str. faecalis</i>	Vancomycin	Kanamycin
C <i>Str. faecalis</i>	Penicillin	Streptomycin
D <i>Str. faecalis</i>	Penicillin	Streptomycin
	Erythromycin	Erythromycin

Organism	Antibiotic	
	Vertical	Horizontal
E <i>P. mirabilis</i> ¹	Polymyxin	BW 56-72
F <i>P. mirabilis</i>	Polymyxin	BW 56-72

¹Test of simple bacteriostatic action without transfer (for further description see text).

FIG. 3. Results of tests by cellophane transfer method (for further description see text).

Organism	Antibiotic		Organism	Antibiotic	
	Vertical	Horizontal		Vertical	Horizontal
A <i>Str. faecalis</i>	Tetracycline	Neomycin	E <i>Staph. pyogenes</i>	Penicillin	Tetracycline
B <i>Staph. pyogenes</i>	Penicillin	Erythromycin	F <i>Str. pneumoniae</i>	Penicillin (250 µg./ml.)	Chloramphenicol
C <i>Staph. pyogenes</i>	Methicillin	Novobiocin			
D <i>Staph. albus</i>	Novobiocin	Penicillin			

to the right of the position of the strip, where the concentration is optimum. Diffusion of streptomycin into this zone converts this partial to total bactericidal action, again to such a distance that the concentration of streptomycin required for the effect is evidently low. Figure 2D (see text, Case 7) is the same test, but with erythromycin also included in both strips, showing that this antibiotic antagonizes both the individual and the combined action of the other two.

Figures 2E and 2F illustrate the combined action of polymyxin and BW 56-72, which is 2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine, an antibacterial substance now undergoing investigation and kindly furnished to us by Dr. D. A. Long of the Wellcome Foundation. Figure 2E is not a cellophane transfer preparation, but a simple nutrient agar plate inoculated with *Proteus mirabilis* after the two substances had diffused from strips, and therefore illustrates only bacteristatic action. Polymyxin (vertical strip) is inactive alone, but is potentiated by BW 56-72, which is also itself bacteristatic. Figure 2F is a cellophane transfer test of the same combination, and shows that a bactericidal effect is only produced where BW 56-72 is diffusing into the polymyxin area. Although this combination, by reason of including a synthetic drug, is strictly outside the present subject, it is included because such a picture is unique in our experience.

Antagonism A minor degree of antagonism (denoted 'a' in Tables I to III), which is interference with the action of neomycin by tetracycline on *Str. faecalis*, is shown in Fig. 3A. Instead of forming a right angle, the zone of profuse growth forms a pointed peripheral extension. (The rash of plaques in this preparation is due to imperfect contact between the cellophane and the medium.) In Fig. 3B penicillin is only partially bactericidal for a (penicillinase-forming) strain of *Staph. pyogenes*: erythromycin, where its concentration is high, is totally bactericidal, but where its concentration is only inhibitory and penicillin is also present, there is a band of profuse growth. This is antagonism at only a certain level of concentration of one of the antibiotics. Figure 3C shows a higher degree of the same effect. Novobiocin is totally bactericidal for this strain of *Staph. pyogenes*: methicillin at optimal concentration (exemplifying well the paradoxical zone phenomenon) is almost totally so, but where it meets a concentration of novobiocin below the bactericidal level for that antibiotic, there is a broad zone of profuse growth. In Fig. 3D (penicillin and novobiocin vs. *Staph. albus* from a case of chronic septicaemia arising in a Spitz-Holter valve) two antibiotics independently bactericidal are shown to be antagonistic by a band of growth, the curvature

of which must presumably mean that the relative proportions for maximum antagonism vary somewhat with absolute concentration.

Figure 3E illustrates antagonism between tetracycline and penicillin acting on *Staph. pyogenes*. Each is partially bactericidal, penicillin much more so, but between them is a very broad zone of profuse growth, indicating antagonism over a wide range of relative concentrations. Figure 3F shows antagonism between chloramphenicol and penicillin acting on a pneumococcus. Chloramphenicol alone is totally bactericidal, and penicillin appears to be so at an optimal (low) concentration, but where they meet there are many survivors. This strain of pneumococcus was cultivated from the cerebrospinal fluid of a patient whose meningitis was initially treated with both of these antibiotics to cover the possibilities of both Gram-positive and Gram-negative infection. As soon as the pneumococcus was identified (and before any such tests as this were done) treatment was continued with intramuscular penicillin alone, with satisfactory immediate results.

The two following cases illustrate the practical value of such tests.

Case 7 Mrs. A.K., aged 48, was admitted to the Royal West Sussex Hospital under the care of Dr. J. D. Whiteside on 13 January 1961 with a history of fever for six weeks, a mitral lesion, and a positive blood culture (*Str. faecalis*). On the basis of sensitivity tests she was treated with penicillin (for most of the time at the rate of 2 mega units four-hourly, reinforced with benemid), streptomycin 0.5 g. b.d., and at first also with erythromycin 500 mg. q.i.d. The culture was referred to us simply for an opinion on whether erythromycin should also be given. As the result of tests to be described it was stopped half way through the six weeks' course: the patient made a good recovery and remains well.

Figure 2C shows the synergic action of penicillin and streptomycin on the streptococcus. The effect of adding erythromycin to this combination was studied in two ways. When a cup containing erythromycin solution was placed near the meeting point of the antibiotic strips, there were numerous survivors in this area. When erythromycin was added to each antibiotic in both strips (Fig. 2D) total bactericidal action was inhibited over the whole area of the plate. It is clear, therefore, that erythromycin can be antagonistic to this combination. A possible previous example of this was reported by Dormer (1960): a girl aged 19 with a *Staph. pyogenes* infection of a patent ductus arteriosus shows no sign in the chart reproduced of responding to the triple combination, but responded promptly to penicillin and streptomycin, shown by test to act synergically on this staphylococcus.

Case 8 J.N., a woman aged 42, with a history of rheumatic fever at the age of 10, was found in November 1960 to have an endocarditis due to *Str. faecalis* involving the aortic valve. After ineffectual treatment elsewhere with penicillin and chloramphenicol she was admitted to the National Heart Hospital under the care of Dr. Paul Wood on 31 January 1961. The streptococcus, re-isolated there in four separate cultures, was inhibited by 4 and 32 µg./ml. respectively of penicillin and streptomycin, but this combination, tested by cellophane transfer, was not totally bactericidal. Of eight other combinations so tested, two were totally bactericidal, penicillin + kanamycin and streptomycin + novobiocin. The use of the former was advised, and for six weeks the patient had penicillin, 2 mega units four-hourly, accompanied by kanamycin, 0.25 g. t.d.s., for 13 days and thereafter 0.166 g. four-hourly. Blood assays were done to verify that this dose was producing adequate blood levels. She recovered completely, and suffered no loss of hearing.

TESTS OF MULTIPLE STRAINS BY CELLOPHANE TRANSFER METHOD This laborious series of tests was done to determine whether this method gives more consistent results than others. All the organisms used were clinical isolates from infections, mostly recent. The largest number of tests was done with the two following 'problem' bacteria.

Staphylococcus pyogenes All the 12 strains used were penicillinase formers, but with the exception of three also resistant to streptomycin and to tetracycline, they were sensitive to all other antibiotics. The results are given in Table I. Where fewer than 12 strains were tested, it is because the combination, e.g., tetracycline + chloramphenicol, was expected to be indifferent. It will be seen that the results are not consistent; with few exceptions they are no more so than others have reported using different

methods. No explanation can be offered for this: it has simply to be accepted that in any critical situation an *ad hoc* test of combined action on the patient's organism must be done. Results of the present kind can only show which combinations are worth testing and which are not. The most noteworthy feature of these results is the very strong antagonism between methicillin and the 'static' antibiotics, much more consistent than the corresponding findings with penicillin. Indeed methicillin + chloramphenicol is the only combination in the whole table which was invariably antagonistic.

Streptococcus faecalis (Table II). All of the 10 strains tested were from cases of endocarditis. The results differ from those obtained with staphylococci in several ways. The usual synergy between penicillin and streptomycin is shown: other combinations with penicillin are more often antagonistic, including erythromycin and vancomycin. The few tests with methicillin again show antagonism not only with static antibiotics but also with vancomycin. Several other combinations differ in their action from that on staphylococci, usually in the direction of being more often antagonistic.

Escherichia coli Tests were done with a total of seven strains, and naturally with a more limited range of antibiotics. The noteworthy feature in these results (Table III) is that, whereas combinations of static antibiotics (tetracycline and chloramphenicol) with two of the bactericidal antibiotics (streptomycin and kanamycin) are often antagonistic, tetracycline and chloramphenicol are usually synergic with polymyxin.

Other bacteria Tests were done with six strains of *Haemophilus influenzae*. They showed synergy

TABLE I
RESULTS OBTAINED BY CELLOPHANE TRANSFER METHOD WITH MULTIPLE STRAINS OF
STAPHYLOCOCCUS PYOGENES

	Methicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin	Vancomycin	Kanamycin
Penicillin	OOOOO OOaaa	OOOOOO OOOOOS	OOOOOO AAAAA	OOOOOO OAAAAA	OOOOOO OOOaaa	OOOOOO OOOaaa	OOOOOO OOOOSS	OOOOOO OOOOOS
Methicillin		OOOOOO OOOOOa	OAAAA AAAAA	AAAAAA AAAAAA	OOaaaa aaaaAA	OOOaaa aaAAAA	OOOOOO OOOOSS	OOOOOO OOSSSS
Streptomycin			OOOOO Oaaaa	OOOOOO OOaaaa	OOOOOO OaaaaS	OOOOOO OOaaaaS	OOOOOO OOOOOS	OOOOOO OOOOOO
Tetracycline				OOOOOO	OOOOOO	OOOOOO a	OOOOaa aaaaA	OOOOOO OaaaA
Chloramphenicol					OOOOO Oaaaa	OOOOO OOaa	OOaaaa aaaaA	OOOOOa aaaaA
Erythromycin						OOOOO OOaa	OOOOO OOOaa	OOOOOO OOaaaa
Novobiocin							OOOOOO OOOOaa	OOOOOO OOOSSS
Vancomycin								OOOOOO OOOOOO

O = indifference

S = synergy

a = antagonism

A = more pronounced antagonism

TABLE II
RESULTS OBTAINED BY CELLOPHANE TRANSFER METHOD WITH MULTIPLE STRAINS OF
STREPTOCOCCUS FAECALIS

	Methicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin	Vancomycin	Kanamycin
Penicillin	Not done	OOSS SSSS	OOOO OaAAA	OAAAA AAAAA	OOaaA AAAAA	OOOOO OOOOa	OOOOa AAAAA	OOOOS SSSSS
Methicillin		SS	AA	AAA	AA	Oa	AAA	SSS
Streptomycin			OOOOO Oaaaa	OOOOA AAAAA	OOaaA AAAA	OOOOO SSSA	OOOOS SSSSS	OOOOO OOOOO
Tetracycline				OOO	OOOO	OOOO	OOOO OOOO	OOOOO OaaAA
Chloramphenicol					OOO	OOOO	OOOOO OOaaa	OAAAA AAAAA
Erythromycin						OOOO	OOOOO OOOaA	OAAAA AAAAA
Novobiocin							OOOOO OOOOa	OOOOO OOOSS
Vancomycin								OOSSS SSSSS

O = indifference S = synergy a = antagonism A = more pronounced antagonism

¹Some of these tests were done with neomycin in place of kanamycin: these two antibiotics behave very similarly.

TABLE III
RESULTS OBTAINED BY CELLOPHANE TRANSFER METHOD
WITH MULTIPLE STRAINS OF *ESCH. COLI*

	Tetracycline	Chloramphenicol	Kanamycin	Polymyxin
Streptomycin	OOOa aaS	aaaa aaa	OOOO OOO	OOO OOO
Tetracycline		OO	Oaa aaa	OOO SSS
Chloramphenicol			Oaa AA	OOS SSS
Kanamycin				OOO OOO

O = indifference S = synergy
a = antagonism A = more pronounced antagonism.

between either penicillin or methicillin and either streptomycin or kanamycin, antagonism between penicillin or methicillin and tetracycline, and consistent indifference between penicillin or methicillin and chloramphenicol. On the other hand chloramphenicol + tetracycline was twice synergic, the only example seen of synergy between two static drugs. Two strains each of *Proteus mirabilis* and *Proteus vulgaris* were tested with most combinations of nine antibiotics. The usual antagonism between static (tetracycline and chloramphenicol) and bactericidal (streptomycin and kanamycin) was found. There were very few examples of synergy (but see Figs. 2E and 2F).

DISCUSSION

PRESENT-DAY APPLICABILITY OF THE JAWETZ LAW
No attempt can be made adequately to review the now extensive literature on this subject, but some

new developments are perhaps worth noting. It is admitted that no combination will invariably behave in the same way, but in so far as its action is reasonably consistent, how far does the simple law formulated by Jawetz and Gunnison (1952) still apply?

It has been evident for some time that there are frequent exceptions to this, and an attempt has now been made by Manten and Wisse (1961) to elaborate the law to cover them. They propose that each class of antibiotic, bactericidal and bacteristatic, requires subdivision. In one class of the static are those, including penicillin and vancomycin, which kill only growing bacteria and are therefore antagonized by certain of the static (tetracyclines, chloramphenicol, etc.). In the other are those lethal to resting bacteria, and therefore not impeded by stasis due to another agent: this class is said to include polymyxins, streptomycin, and neomycin. This distinction must be agreed for polymyxin. Manten and De Nooy (1956, 1959) have previously shown that polymyxin and chloramphenicol act synergically on species of *Salmonella*, the opposite of what is to be expected according to the Jawetz law. This action is verified, and supported by the behaviour of polymyxin + tetracycline, in the few tests with *Esch. coli* reported here (Table III). On the other hand, the present results do not support the idea that streptomycin should be placed in this category, and the underlying assumption that it is bactericidal to resting cells is incorrect (Garrod, 1948). In so far as neomycin and kanamycin can be equated, the same argument applies to neomycin. Manten and Wisse also subdivide static drugs, proposing a small category, including cycloserine (and sulphonamides), which do

not antagonize penicillin, etc., 'because a comparatively long space of time elapses before these drugs cause bacteriostasis' and meanwhile 'irretrievable injury' can be done by the cidal component.

We believe that a further and perhaps more important step can be taken towards reconciling practical findings with the Jawetz law by recognizing that some antibiotics have a dual type of action. Erythromycin and novobiocin, in particular, can be static in lower concentrations and cidal in higher, and the nature of combined action will depend on which of these is at work. The cellophane transfer method brings this out well: it is clear in Fig. 3B that erythromycin antagonizes penicillin only in a static concentration. Even chloramphenicol may be cidal in a high concentration (Fig. 3F), and we have other evidence that it is cidal at much lower levels for *H. influenzae*, a highly sensitive organism. If this additional factor is taken into account, and a few antibiotics, certainly including polymyxin and other polypeptides, are excepted altogether, the Jawetz law appears still to be generally applicable.

CLINICAL UTILITY OF TESTS OF COMBINED ACTION The purpose of citing a series of case histories here is to show that tests of combined bactericidal action are a reliable guide to effective treatment. These histories are not exceptional: successful instances, particularly of the penicillin-streptomycin combination for *Str. faecalis* infections, could be multiplied. Failures have been due either to the advanced stage of the disease when the recommended treatment was adopted, with such gross valvular deformity as to render heart failure inevitable, or to an infection (as in Case 1) due to an organism for which no effective combination could be found. If a combination can be identified which is totally bactericidal for a heavy inoculum, sterilization of the lesion can confidently be expected if a full course of treatment is not interrupted by death or intolerance to the drug.

Of the two methods described, that employing a liquid medium with subculture is easier to perform, and if applied only to a few likely combinations, should indeed be within the capacity of almost any clinical laboratory. The necessary effort to carry it out for the very few serious problem cases which occur annually in any hospital should surely be made. The cellophane transfer method is more difficult and requires special apparatus, experience, and more time. Its advantage is that it gives a picture of both individual and combined action at different absolute and (in combined action) relative concentrations. How closely the details so revealed

can be interpreted in therapeutic terms further experience will be necessary to decide.

We are indebted to the physicians named in the text for permission to refer to their patients. Much of the work described has only confirmed the findings of Dr. Y. Chabbert, who originated both of the methods used, but one of us (P.M.W.) is further indebted to him for the hospitality of his laboratory and for kind instruction in the cellophane transfer technique. This visit was made possible by a study grant from the Institute of Medical Laboratory Technology, to which we also express our thanks.

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ADDENDUM

This paper was written before we had seen that by R. Tompsett and M. Pizette (*Arch. int. Med.*, 1962, **109**, 146) describing four cases of enterococcal endocarditis, all of which responded to penicillin + streptomycin, although this combination was totally bactericidal *in vitro* for only two of the strains of enterococcus. We do not agree with their conclusion that such tests 'are not of value in predicting the usefulness' of this treatment, but in view of these therapeutic results this combination should evidently be tried, despite an unfavourable result *in vitro*, if no better one can be found. It is noteworthy in this connexion that according to E. Jawetz *et al.* (*J. gen. Microbiol.*, 1954, **10**, 191) streptomycin may act synergically with penicillin on *Str. faecalis* in as little as 1/1000th of the concentration required for its independent effect.