LABORATORY ASPECTS OF COMBINED ANTIBIOTIC TREATMENT

BY

S. D. ELEK, M.D., Ph.D., D.P.H.

G. R. F. HILSON, M.D.

AND

PAMELA JEWELL, M.B., B.S.

(From the Department of Bacteriology, St. George's
Hospital Medical School, London)

Since Ungar's observation in 1943 that penicillin and sulphonamides enhance each other's action, the subject of synergism and antagonism between antibacterial substances has received much attention and has recently been extensively reviewed by Garrod (1953). Tests have been made both *in vitro* and *in vivo*, and it is certain that some interaction between antibiotics exists and is likely to be therapeutically important. Clinical experience, though limited, confirms this view; on the one hand, it appears that in the treatment of enterococcal endocarditis a combination of penicillin and streptomycin is more efficient than either drug alone (Hunter, 1950; Robbins and Tompsett, 1951; Cates *et al.*, 1951), and, on the other hand, in pneumococcal meningitis, penicillin and aureomycin used together are less effective than penicillin alone (Lepper and Dowling, 1951).

Interaction occurs in at least three of the manifestations of the antibiotic action: inhibition of growth, lethal effect, and emergence of resistant variants. Growth-inhibition tests are simple to perform, but when used to investigate the combined action of antibiotics often fail to correlate with findings *in vivo* (Jawetz, Gunnison, Speck, and Coleman, 1951; Chopra and Gupta, 1951; Bliss *et al.*, 1952). The estimation of the bactericidal effect of a pair of drugs, as opposed to bacteriostatic effect, is much more laborious, but interaction shown in this way may have more therapeutic significance (Hunter, 1950; Gunnison, Jawetz, and Coleman, 1950; Garrod, 1953). The emergence of resistant variants is also bound up with bactericidal action. The present paper records an investigation of the lethal action of combinations of antibiotics, using a technique simpler than those previously described. The terms synergism and antagonism are defined as follows: a combination of drugs is synergistic when its lethal effect is greater than that of the more lethal drug alone, and antagonistic when its effect is less. Synergism, defined in this way, includes the term "additive" described by other authors. There may, of course, be no interaction, in which case the effect of the more lethal drug is dominant.

A number of different methods have been used in studying the bactericidal effects of mixtures of antibiotics. Generally, complete viable counts have been made at intervals on cultures containing several different concentrations of antibiotics. In order to carry out testing on a larger scale, some workers have used simplified viable counts (Bigger, 1944; Nichols, 1948; Rantz and Randall, 1952), and others the turbidimetric estimation of growth from surviving bacteria (Thomas and Hayes, 1947; Burnell and Kirby, 1951). All these methods are unsuitable for routine testing in a busy clinical laboratory. The simplified viable count method of Martin *et al.* (1952), recommended by Garrod (1953), tests the

action of a pair of antibiotics at *one* concentration only. This is likely to be misleading, as there are reports that concentrations are important (Hobby and Dawson, 1946; Bliss *et al.*, 1952). The conditions under which antibiotics act in the body are varied, and it is probable that when a patient receives two drugs their relative concentration fluctuates widely.

Agar diffusion methods used to detect inhibitory levels have the great advantage of allowing a range of relative concentrations to be tested easily, and as they are also widely employed for routine sensitivity tests it is not surprising that attempts have been made to show interaction by such methods (Peyré and Velu, 1952; King *et al.*, 1953; Garrod, 1953). These attempts have, however, been based on interactions at the inhibitory level, and we believe that the appearances of inhibition on solid media can give misleading information. A further step is required to show whether the growth one sees is alive or whether in fact it has been killed. The technique we have used is an agar diffusion method amplified by a simple form of sampling to reveal the lethal action of the antibiotics.

Method

The method used is the one described in detail by Elek and Hilson (1953), and consists of a transfer of organisms from the surface of the original medium containing antibiotics to the surface of a fresh medium free from drugs. The transfer is made in much the same way that a rubber stamp transfers ink from a pad to a piece of paper: the stamp consists of a cylindrical block of wood with furnish velvet glued to one surface.

Plates were prepared in a manner similar to the paper disk method of sensitivity testing described by Fairbrother and Martyn (1951). Disks (6 mm. in diameter) were cut out of Ford 428 white blotting-paper with an office punch. The concentrations of the antibiotics used were: penicillin G 500 units per ml., except for enterococci, when a concentration of 50,000 units per ml. was used; streptomycin, 1,000 µg. per ml.; chloramphenicol, 5,000 µg. per ml.; and aureomycin, 12,500 µg. per ml. A well-dried agar plate was flooded with a young broth culture which was just faintly turbid and provided the smallest inoculum capable of producing a confluent growth.

The test for antagonism was set up as follows. Three equidistant points were chosen on the inoculated plate (Fig. 1a). The first received a disk saturated evenly with antibiotic A, the second received a disk similarly treated with antibiotic B, while on the third point two similar disks (A and B) were superimposed. Synergism was tested by placing two disks close enough together to produce overlapping of their zones of inhibition (Fig. 2a). After 18 to 24 hours' incubation at 37° C., the appearances due to inhibition of growth were noted. A velvet stamp, previously sterilized by autoclaving, was then pressed evenly on the surface of the plate (the "primary" plate) and then pressed similarly on the surface of a sterile plate (the "replica" plate) which was incubated overnight.

Interpretation

The appearances of *inhibitory* effects on the primary plates could not be interpreted reliably. For example, "tongues" of growth were often seen between two disks, suggesting antagonism, but the replica plates showed that sometimes the "tongues" contained no living organisms. Furthermore, areas free from growth were frequently shown to contain many viable bacteria.

The appearances of *bactericidal* interaction which the replica plates revealed were, however, clear-cut. Typical findings on primary and replica plates are illustrated. Fig. 1 shows what is seen when two drugs are antagonistic: the

zones of inhibition produced by drugs A and B on the primary plate are similar, but the replica plate shows that A has killed all the organisms within its zone, while B has allowed almost complete survival. The inhibition zone due to A and B together on the primary plate resembles that of either alone, which would suggest that there has been no interaction. However, the replica shows that the expected lethal action of A is absent, and therefore the combination of A with B is antagonistic. Synergism is illustrated in

(a) PRIMARY PLATE

(a) PRIMARY PLATE

(b) REPLICA PLATE

FIG. 1.—Antagonism.

(b) REPLICA PLATE

FIG. 2.—Synergism.

Fig. 2. The bactericidal action of A extends beyond its expected radius in the area where C is also present. In other words, a sublethal concentration of A becomes lethal when the organisms are also affected by C. This is also shown by the flattening of the contour where the zone edges meet.

Results

The methods just described were used, and 65 strains of common pathogens were tested with three pairs of antibiotics: penicillin with chloramphenicol, penicillin with aureomycin, and streptomycin with chloramphenicol. The organisms consisted of 28 strains of *Staph. pyogenes* from human lesions, 17 strains of *Bact. coli* from urinary infections, 10 strains of enterococci from cases of subacute bacterial endocarditis, and 10 strains of haemolytic streptococci (Groups A, B, and C). The results are summarized

Interactions After 18 to 24 Hours

Organism	No.	Penicillin + Aureomycin			Penicillin + Chloramphenicol			Streptomycin + Chloramphenicol		
		S.	N.I.	A.	S.	N.I.	A.	S.	N.I.	A.
<i>Staph. pyogenes</i>	28	0	4	24	0	9	19	21	7	0
Haem. strep.: Group A ..	5	0	3	2	0	2	3	0	5	0
" B ..	3	0	1	2	0	1	2	1	2	0
" C ..	2	0	0	2	0	0	2	2	0	0
" D ..	10	0	4	6	0	1	9	4	6	0
(endocarditis)										
<i>Bact. coli</i> (urinary) ..	17	0	14	3	0	17	0	10	7	0
Total ..	65	0	26	39	0	30	35	38	27	0

S.=Synergism. N.I.=No interaction. A.=Antagonism.

in the Table, from which some consistency in behaviour can be seen for each pair of antibiotics. The combination of penicillin with either chloramphenicol or aureomycin was antagonistic, or showed no interaction, while streptomycin with chloramphenicol was synergistic, or showed no interaction. These general rules appear to be broadly independent of the species of organism.

It must be stressed that the results were obtained by incubating the primary plates for 18 to 24 hours before replica plating. Jawetz, Gunnison, and Speck (1951), using one strain of *Str. pyogenes* and one strain of *Klebsiella pneumoniae* (*Bact. friedländeri*), reported that streptomycin and chloramphenicol were synergistic after 24 hours' incubation, but antagonistic during the first 12 hours. The earlier findings correlated best with their *in vivo* results, which led them to conclude that they may be more significant therapeutically. We therefore retested six strains of staphylococci and five strains of *Bact. coli* after six to eight hours' incubation of the primary plates. Tested in this way, streptomycin with chloramphenicol was antagonistic with all six strains of staphylococci and with four out of the five *Bact. coli* strains, which contrasts with the synergism found after overnight incubation. Penicillin with aureomycin, tested at the earlier time, showed antagonism or no interaction, the same as was found at 18 to 24 hours' incubation.

Discussion

On the whole the findings with the method presented here agree with those obtained by other techniques. The antagonism of penicillin and aureomycin has been shown with several different organisms (Gunnison, Coleman, and Jawetz, 1950; Gunnison, Jawetz, and Coleman, 1950; Hunter, 1950), and the antagonism of penicillin with chloramphenicol has also been previously reported (Jawetz, Gunnison, Speck, and Coleman, 1951; Burnell and Kirby, 1951). These two combinations were, however, found to be synergistic with one strain of *Salm. typhi*

(Price *et al.*, 1949) and one strain of *Proteus vulgaris* (Jawetz *et al.*, 1952). It has also been suggested that the results depend on the concentrations of the drugs used (Bliss *et al.*, 1952).

Streptomycin and chloramphenicol have been reported as synergistic with 6 out of 33 strains of *Staph. pyogenes*, there being no interaction with the remainder (Rantz and Randall, 1952). Other workers have obtained variable results according to the organism tested or the concentration of drugs used (Jawetz *et al.*, 1952; Bliss *et al.*, 1952). In general, our results agree with those of Jawetz, Gunnison, and Speck (1951), in that this combination was often synergistic after 18 hours' incubation, though antagonistic during the first few hours.

Jawetz and his associates have come to some general conclusions about the behaviour of antibiotic interaction (Jawetz *et al.*, 1952). They believe that antibiotics may be grouped into bactericidal (group 1) and bacteriostatic (group 2) drugs, and suggest that pairs of group 1 are synergistic, while group 1 and group 2 drugs together are antagonistic. They point out, however, that this is only a general rule, to which there are exceptions, and that results for a given organism with any pair of antibiotics cannot be forecast. Hitherto the number of bacterial strains tested has been severely limited by the laborious nature of the techniques required. As a result of our findings with a comparatively large number of strains of different species of bacteria, we can confirm that group 1 and group 2 drugs may be antagonistic during the early period of their action together. With continued incubation up to 24 hours, penicillin and aureomycin may remain antagonistic or may no

longer show interaction; under the same conditions streptomycin and chloramphenicol not only lose their initial antagonism, but with some organisms become synergistic. Unlike some workers (Jawetz *et al.*, 1952; Bliss *et al.*, 1952), we have not found at 18–24 hours that a pair of antibiotics will be synergistic with one organism and antagonistic with another. In our experience any pair of antibiotics will react at this time in the same way, varying only in degree with different organisms. We therefore believe that a limited forecast can be made of the behaviour *in vitro* of a pair of antibiotics when tested against any organism. The value of such a forecast in controlling therapy cannot yet be assessed, since further knowledge of the correlation between laboratory and clinical findings is needed.

Before any prediction can be made of the probable correlation between laboratory and clinical results several difficult questions have to be considered. The first is the problem of the relative importance of bacteriostatic and bactericidal action of antibiotics in the body. The paramount importance of the former mode of action in most forms of infection was not questioned until the combined action of antibiotics was investigated. It then became apparent that the results of testing bacteriostatic activity *in vitro* agreed less well with animal experiments than did the results of tests based on bactericidal action, and the idea has grown up that the lethal activity of antibiotics is the more important *in vivo*. Concentrations used in tests of combined lethal activity have, however, been closer to those attained in the body than the sub-inhibitory levels usually employed in tests based on combined bacteriostatic action, and this may explain some of the divergent results. There can be no doubt that the pattern of interaction alters with time. Jawetz and his colleagues suggest that early readings are of clinical importance, although other workers came to the opposite conclusion (Ahern *et al.*, 1952). Since antibiotic treatment is usually prolonged, we feel that results after 24 hours' contact with the drugs are more likely to be significant. It may even be that results observed after still longer periods are of greatest value. Finally the synergistic lethal action of two antibiotics by allowing fewer survivors may lessen the chance of the emergence of resistant variants. There can be no definite answer to these questions until more work is done to correlate *in vitro* and clinical findings. The method described here may help to serve this purpose.

Summary

There is both experimental and clinical evidence that two antibiotics used simultaneously may be more efficient (synergistic) or less efficient (antagonistic) in their action than either drug used alone.

A simple method suitable for routine diagnostic work is described. It reveals interactions of the bactericidal effect of antibiotics *in vitro*. Sixty-five strains of common pathogens were tested against three pairs of antibiotics. Penicillin with either chloramphenicol or aureomycin generally showed antagonism. Streptomycin with chloramphenicol was synergistic. These results refer to interaction after 18 to 24 hours. The only exceptions to these findings were that no interaction occurred.

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CLINICAL TRIAL OF METHIMAZOLE IN TREATMENT OF THYROTOXICOSIS

BY

LINDSAY A. G. DAVIDSON, M.B., Ch.B.

Medical Registrar, Dundee Royal Infirmary; formerly
Medical Registrar, Dryburn Hospital, Durham

The history of the drug treatment of thyrotoxicosis dates from Chesney's discovery in 1928 that rabbits fed exclusively on cabbage became myxoedematous and developed goitre.¹ Thiouracil was introduced to clinical medicine in 1943 in a paper by E. B. Astwood,² and in 1949 Stanley and Astwood³ reported that 1-methyl-2-mercaptoimidazole, now known as methimazole, was the most active antithyroid drug they had encountered. Methimazole has a basic ring structure of five atoms. This ring contains two nitrogen atoms connected through a carbon atom to which is attached a sulphur atom. This chemical grouping —N—CS—N—, which is common also to the six-membered ring of the thiouracils, is thought to be responsible for the antithyroid activity of the drug (see Formula).

1-Methyl-2-mercaptoimidazole
(Methimazole)

2-Thiouracil

Formula of methimazole compared with that of thiouracil.

The normal thyroid gland selectively removes iodine from the blood plasma and converts it into thyroxine. This function is inhibited by antithyroid drugs, which block the formation of di-iodotyrosine within the gland.³⁸ Using the degree of block produced as an index of antithyroid activity, Stanley and Astwood have estimated in man the ability of the different antithyroid drugs to block, in the gland, the conversion into thyroxine of a test dose of radioactive iodine, and thus estimated the clinical potency of each drug.⁴ In experiments of this type they found methimazole one hundred times more effective weight for weight than thiouracil.³ The actual figures published are set out in Table I.

In general clinical use methimazole is 20–50 times more effective weight for weight than compounds of thiouracil. In man a single dose of 5 mg. of methimazole almost completely inhibited the accumulation of radioactive iodine by the gland for nearly 24 hours.³ In