THE TESTING OF ANTISEPTICS IN RELATION TO THEIR USE IN WOUND TREATMENT[1].

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In previous reports (Browning, Gulbransen, Kennaway and Thornton) attention was directed to the value of certain experimental tests of antiseptics as an indication of their practical value in the treatment of infected wounds, namely the estimation of antiseptic potency in the presence of serum and the relationship between this property and toxicity to tissues. In regard to the latter factor it was pointed out that toxicity must be investigated on a wide basis; thus determinations were made of the effect of antiseptics on leucocytes, as shown by alterations in their phagocytic properties, and on an epithelial membrane, *e.g.* the conjunctiva, as evidenced by the production of inflammation, and also the toxicity to the body as a whole was estimated in cases where the substance was readily absorbed (see Table I). It was emphasised,

Table I.

Substance	Maximum non-lethal dose for 20 gram mouse	Bactericidal concentration for *Staph. aureus* in 0·7 % peptone water	*Staph. aureus* in Serum	*B. coli* (Escherich) in 0·7 % peptone water	*B. coli* (Escherich) in Serum
Diamino-acridine sulphate (proflavine)*	0·003 gram	1 : 20,000	1 : 200,000	1 : 4,000	1 : 100,000
Diamino-methyl acridinium chloride (acriflavine)*	0·0006 „	1 : 20,000	1 : 200,000	1 : 1,300	1 : 100,000
Phenol	0·006 „	1 : 250	1 : 250	1 : 500	1 : 500
Mercury perchloride	0·0001 „	1 : 1,000,000	1 : 10,000	1 : 1,000,000	1 : 10,000

Method of the Tests. The toxicity for mice was determined by injecting watery solutions subcutaneously, the dose being so arranged that 20 grm. mouse received a volume of 1 c.c.; to animals of other weights corresponding volumes were given, but mice not exceeding the limits of 15–25 grm. were selected for the tests.

* It has been suggested that the increased bactericidal action of the flavines in serum is merely an instance of a general enhancement of toxicity due to serum; as might be anticipated the injection of the dose in 80 per cent. serum instead of a watery solution does not, however, alter the fatal dose.

however, that the susceptibility of different tissues toward a given chemical compound may vary greatly and that this important character in substances destined for use as antiseptics requires much further investigation; the impossibility of using strychnine, even if it were a potent antiseptic, owing

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to its specialised toxic action on the nervous system was cited. The result of our work on these lines pointed to the value of certain basic benzol derivatives —*brilliant green*—and acridine compounds—*acriflavine* and *proflavine*. Independent clinical observations (Ligat, James) supported the view that the sum total of tests which we had applied, constituted a trustworthy guide to the therapeutic value of these antiseptics and afforded indications as to the suitable means of application. In the interest of further progress it was obviously of great importance that the significance of suitable laboratory tests should be established as a guide to practical use, since such investigations constitute an essential preliminary to clinical trials. Subsequent observations by Drummond and McNee, Carslaw, Carslaw and Templeton, and Pilcher and Hull, have added greatly to our knowledge of the therapeutic value of the flavine compounds both for prevention and treatment of sepsis, and the clinical material on which the reports are based (over 5000 cases in Pilcher and Hull's series) indicates the representative nature of the tests. From these investigations the result emerges that, employed in conjunction with operative measures, salts of certain basic organic compounds, especially the flavines and also brilliant green, are capable of yielding valuable therapeutic results when applied by the relatively simple and rapid method of packing with gauze soaked in—not wrung out of—a solution of the antiseptic and an important feature of the flavines is that infrequent renewal in the wound suffices to maintain their effect. This may be advantageously provided for, on occasion, by a valved tube according to Kellock's method. The satisfactory action of infrequent renewal of the antiseptic seems clearly to be correlated with the continued activity of the flavine compounds in the presence of serum. The importance of interrupting the application of flavine after a certain stage, owing to an inhibition of healing—not to any destructive action on existing tissues—which may supervene, is a valuable practical contribution to our knowledge of the use of these compounds which has been established by the above-named clinical observers (see also Bashford, Hartley and Morrison). Several investigators, however, have published results of laboratory investigations tending to the conclusion that we have exaggerated the properties of the flavine compounds; accordingly, a number of points which have been raised relative to the action of these antiseptics on bacteria will be dealt with here. Their action on the tissues will be discussed elsewhere.

THE CHOICE OF A MEDIUM FOR TESTING ANTISEPTICS.

Serum was originally selected because serous exudate constitutes practically the diluent to which an antiseptic is exposed in a wound, both when recent and, also at a later stage when a granulating surface is mechanically cleansed from pus. The fact that serum intensifies the bactericidal action of the flavines, especially in the case of *B. coli*, has been met by the statement that serum is a poor culture medium and it has been suggested that the intensifying effect is merely a summation of the inhibitory properties of serum

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and of the antiseptic. The error of these views becomes clearly apparent when one tests a number of specimens of serum and also a variety of antiseptics. Thus, while some specimens of ox serum afford a relatively poor medium for the proliferation of *B. coli*, others yield excellent growths. On what this depends has not been determined; the unsuitable sera are usually those which are practically colourless; but the difference has not been found to depend on factors such as length of contact with the clot or the addition of a trace of laked red corpuscles. The intensifying action of serum on the bactericidal property of flavine is well shown in the case of sera which are favourable to bacterial growth. The addition to the serum of an amount of trypsin (see Douglas and Colebrook) which augments the properties as a medium of unfavourable specimens, has not been found to alter distinctly its intensifying action for flavine[1]. The summation theory is further disproved by finding substances which are extremely potent antiseptics in watery medium, but whose action is greatly reduced by serum, *e.g.* in the case of brilliant green with *B. coli*—

Concentration in 0·7 % peptone water which kills = 1 : 130,000.

Concentration in serum which fails to kill = 1 : 5000 (result obtained by subculture after 48 hours: the control culture in serum without antiseptic yielded abundant growth).

If admixture with serum, in addition to merely diluting the antiseptic, also neutralises its effects, then frequent renewal of the substance will be essential in order to obtain efficient action in a wound. This is the case with all the common older antiseptics in watery solution except carbolic acid; such frequent renewal exposes the body as a whole to the danger of toxic effects should absorption occur; phenol and mercuric chloride are potent poisons when absorbed, hence their applicability is strictly limited. On the other hand, the hypochlorites have the great advantage of being converted into harmless compounds in contact with the tissues, but the "complicated hydraulic system," as Dakin, Lee, Sweet, Hendrix and Le Conte term it, which is necessitated by the unstable nature of these antiseptics in watery solution, in order to obtain efficient action, renders the suggestion of their general use practically a counsel of perfection. Brilliant green (first used by Leitch, see also Ligat and Webb) although diminished in action by serum, is highly potent and is scarcely absorbed; on the other hand, the flavines while absorbed are very little toxic, as was demonstrated by the fact that relatively large amounts could be administered intravenously (intravenous administration has now been practised on an extensive scale in cases of trench fever—Byam, Dimond, Sorapure, Wilson and Peacock).

As regards the action of other media, *pus* diminishes the antiseptic power

[1] Subsequent experiments have shown that the commercial preparation of trypsin which has been recommended for use varies considerably, thus two specimens acted as above stated; a third, however, in similar concentrations produced great alteration of the serum, as shown by abundant formation of tyrosine crystals without any bacterial contamination. Such profoundly altered serum ceased to give a marked intensification of the antiseptic effect of the flavines. This point is being investigated further.

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of the flavines, as was shown in our first report (confirmed by Fleming, and Parry Morgan). *Defibrinated blood* diminishes the action as we demonstrated (confirmed by Fleming, Dakin and Dunham, Parry Morgan).

Milk diminishes the action (Hewlett), so does *minced meat* (Fleming), which also removes the bactericidal properties of blood serum. Dakin and Dunham have used further a mixture of watery muscle extract with serum. It would, of course, be of great value if an antiseptic were to act equally well in all media, but, this is probably impossible and even if an antiseptic could be disseminated in potent concentration by the blood stream it would still fail to penetrate effectively into any considerable mass of necrotic tissue, hence operative interference must be an essential factor in wound treatment. As regards the use of these other media, whose effect in neutralising flavine seems to have been regarded as an important observation by some of the investigators above mentioned, no cogent argument has been adduced to show that, as compared with serum, all or any of them constitute a more rational test medium for ascertaining the value of antiseptics in wounds. Milk and minced meat are clearly of only remote application, while, as we have repeatedly pointed out, pus is not the medium in which an antiseptic is required to act. Before application of an antiseptic pus and necrotic tissue should be removed mechanically; the layer of granulation tissue into contact with which the solution then comes is widely different from pus in its physical characters. We have throughout endeavoured to employ tests which possess "heuristic" value, and consider that the test in serum has been shown to belong to this category, whereas no proof has been afforded that the others do. It has been pointed out that the flavines are compatible with hypertonic saline up to 5 % NaCl; hence a means was provided for regulating to some extent the amount of serous fluid in the wound. This combination of flavine with hypertonic saline has been advantageously employed by Pilcher and Hull.

METHODS OF TESTING ANTISEPTIC POTENCY—BACTERIOSTATIC ACTION IN RELATION TO TOXICITY (EFFECT ON PHAGOCYTOSIS).

We pointed out originally that the flavines and brilliant green bring about the death of bacteria comparatively slowly; but apart from lethal action, very great dilutions of these compounds are effective in checking bacterial multiplication, that is, they exhibit a high degree of *bacteriostatic* action, to use Gildersleeve's term, and so act as potent antiseptics *sensu stricto*. On the other hand, substances such as mercuric chloride, phenol and chloramine-T all produce their maximum effect rapidly (within two hours) and no significant action occurs subsequently, *i.e.* concentrations of the latter substances which fail to prove lethal quickly, exert little or no effect on the multiplication of surviving bacteria. In addition, when it is remembered that serum neutralises these antiseptics—except phenol—the need for frequent renewal becomes clear; but the high toxicity of mercuric chloride and of phenol (which is a

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very weak antiseptic) in relation to their antiseptic potency preclude unrestricted renewal.

It appeared that bacteriostatic action was a most valuable property for wound therapy and, because the serous exudate in the wound did not neutralise the flavines, mere slowness of lethal action as determined *in vitro* mattered little; the organisms under the influence of the antiseptic were, so to speak, controlled from the beginning. A striking confirmation of this is afforded by the observations of Drummond and McNee and also of Carslaw and Templeton, who found that in spite of the presence of bacteria in wounds treated with flavine there is a notable absence of both the local and the general phenomena of inflammation and of septic infections. Experimental evidence bearing on the co-operation of antiseptics with the tissues in overcoming infection will be published elsewhere.

Methods of testing antiseptics *in vitro*, whose sole aim is to detect whether or not every viable organism has been destroyed, may fail altogether to afford a true estimate of the potency of a substance for therapeutic purposes. Thus, Fleming and Hewlett inoculated fluid medium with the mixture of organisms and antiseptic and so obtained no information as to the numbers of organisms which had been killed short of total sterilisation. We have always made subcultures from the antiseptic mixtures on to solid media, so as to determine, by means of the number of colonies, whether the bacteria had increased or decreased. As the inoculation dose of organisms we originally chose a relatively minute number; the medium is not thereby rendered turbid at the time of inoculation, accordingly, if the mixture develops opacity later on, this indicates that free proliferation of the organisms has occurred. It was felt, however, that the use of a minute inoculation exposed our work to the objection that the antiseptic could not inhibit larger numbers of bacteria; accordingly, our second report contained experiments with much larger amounts of organisms, and it was shown in the case of acriflavine and *B. coli* that a twenty-thousand-fold increase in the inoculation dose necessitated only about a two-and-a-half-fold increase in the amount of antiseptic required to produce complete sterilisation. However, without direct reference to this latter work, our results with the basic organic antiseptics have been ascribed by Fleming, Hewlett, and Dakin and Dunham to the use of minute doses of organisms, which have been stated to lead to an exaggeration of the potency of the flavines as compared with other compounds. Accordingly, further tests have been performed, using larger amounts of organisms in conformity with the experiments of Dakin and Dunham.

The following is an example:

A 24-hour agar slope culture of *Staphylococcus aureus* was suspended in 5 c.c. of 0·85 per cent. NaCl solution: 0·025 c.c. each of a 1 : 100,000 and 1 : 1,000,000 dilution of this suspension when plated on agar yielded respectively 175 and 21 colonies, hence it may be concluded that the stock suspension contained about 750 million organisms per cubic centimetre.

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To a series of tubes each containing 1 c.c. ox serum (heated previously for ½ hour at 55° C.) 0·1 c.c. of the *Staphylococcus* suspension was added and also 0·1 c.c. of varying dilutions of the antiseptic in water ("strong inoculation" series). In the "weak inoculation" series the inoculation dose of staphylococci was 0·1 c.c. of a 1 : 1000 dilution of a 24-hour peptone water culture. After incubation of the mixtures of organisms and antiseptic at 37° C. for 24 hours a loopful from each tube was stroked on agar; which was then incubated for 48 hours. The results were as follows:

Table II.

Results of Subculture from Mixtures of Staphylococcus aureus *and Flavines after 24 hours' contact at* 37° C.

Concentration of antiseptic	Acriflavine: Weak inoculation	Acriflavine: Strong inoculation	Proflavine: Weak inoculation	Proflavine: Strong inoculation
1 : 400,000	Marked diminution	—	8 colonies	—
1 : 200,000	Sterile	—	Sterile	—
1 : 130,000	,,	Diminution	,,	Diminution
1 : 100,000	,,	,,	,,	,,
1 : 40,000	,,	Sterile	,,	About a dozen colonies
1 : 20,000	,,	,,	,,	,, ,, ,,
1 : 13,000	,,	,,	,,	,, ,, ,, (Sterile in 48 hours)
1 : 4,000	,,	,,	,,	Sterile

Controls after incubation:

Weak inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 22 colonies.
Strong inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 36 colonies.

Parallel experiments with the *strong* inoculation in a medium containing 33 per cent. serum yielded the same result as those with full serum.

In this and the following table "Diminution" = diminished number of colonies in subculture as compared with control without antiseptic.

In all cases the culture tubes were well shaken before making subcultures, so that any fallacy due to spontaneous sedimentation is excluded.

It might be objected that a loopful was an insufficient sample to take from each specimen; but the fact that a loopful of a 1 : 100,000 dilution of the inoculated serum controls without antiseptic, after incubation, yielded several dozen (22–36) colonies (see also Table III), proves that there is here no fallacy. Since the large inoculation dose, as employed by Dakin and Dunham, imparts a marked turbidity to the mixture, it was considered important to determine the relative number of viable organisms present in the control tubes containing serum, but no antiseptic, not only at the commencement of the experiment, but also after incubation. In order to do this a series of decimal dilutions was prepared in both cases and a loopful from each was stroked on agar; this simple procedure may be recommended as of sufficient accuracy for practical purposes, while it saves time and materials consumed by plating a series of dilutions. The results after incubation of the plates for 48 hours were as follows:

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Table III.

Dilution	Serum *plus* Weak inoculation subcultured at once	Serum *plus* Weak inoculation subcultured after 24 hours at 37° C.	Serum *plus* Strong inoculation subcultured at once	Serum *plus* Strong inoculation subcultured after 24 hours at 37° C.
Undiluted	20 colonies	Homogeneous stroke	Homogeneous stroke	Homogeneous stroke
1 : 10	—	Slight decrease in density of growth	Homogeneous stroke	Discrete but closely adjacent colonies
1 : 100	—	Discrete but closely adjacent colonies	Slight decrease in density of growth	,,
1 : 1,000	—	Marked decrease in number of colonies	Discrete but closely adjacent colonies	,,
1 : 10,000	—	55 colonies	,,	Marked decrease in number of colonies
1 : 100,000	—	22 ,,	,,	36 colonies

The results illustrated in Tables II and III show:

(1) Controls: the large amount of organisms, as used for inoculation by Dakin and Dunham, did not maintain itself, *i.e.* in spite of some proliferation, as evidenced by increase in turbidity of the culture, the large inoculation led to increased death of bacteria, so that viable organisms were more numerous at the beginning than at the end of the experiment. It is obvious, therefore, that this is not the most suitable amount of organisms for testing the properties of a progressively acting antiseptic, since under the conditions arranged by Dakin and Dunham the organisms diminish in the absence of any antiseptic.

(2) With *Acriflavine*, in spite of the enormous number of organisms in the strong inoculation series, the lethal concentration (1 : 40,000) was not more than five times that found with the weak inoculation; further, a concentration of 1 : 130,000 produced a definite lethal effect on the organisms in the strong inoculation series. In the case of *Proflavine* it is clear that the result for practical purposes is the same, but there is a longer range of persistence of viable organisms before complete sterility is attained. Thus a loopful from the *undiluted* mixture containing proflavine 1 : 40,000 yielded only a dozen colonies, whereas the control without antiseptic when diluted 1 : 100,000 yielded 36 colonies in a loopful; therefore there can be no question as to the powerful bactericidal action exerted by this high dilution of the antiseptic. Such an effect will fail to be observed when testing results merely by subculturing into fluid medium, as practised by Fleming and Hewlett, where one viable organism will yield a growth; this would account for their conclusion that our original findings were exaggerated.

The following is a similar experiment in which acriflavine and proflavine were tested with large and small amounts of *B. coli*.

Method as above: the strong inoculation contained about 5000 million more organisms than the weak inoculation. The results are shown in Table IV.

The Control *weak* inoculation subcultured at once yielded 220 colonies in a loopful and after incubation 1 : 10,000, 1 : 100,000, and 1 : 1,000,000 dilutions yielded 65, 30 and 2 colonies in a loopful respectively.

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Table IV.

Results of Subculture from Mixtures of B. coli *and Flavines after 24 hours' contact at 37° C.*

	Acriflavine		Proflavine	
Dilution	Weak inoculation	Strong inoculation	Weak inoculation	Strong inoculation
1 : 400,000	Growth	—	Diminution	—
1 : 200,000	A few colonies (sterile in 48 hours)	—	A few colonies (sterile in 48 hours)	—
1 : 100,000	do.	Growth	Sterile	A few colonies (sterile in 48 hours)
1 : 40,000	—	A few colonies (sterile in 48 hours)	,,	Sterile
1 : 20,000	—	,,	,,	,,
1 : 13,000	—	Sterile	,,	,,

Controls after incubation:

Weak inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 30 colonies.

Strong inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 1 colony.

Parallel experiments with 33 per cent. serum medium gave the same result as full serum in the case of the strong inoculation.

The Control receiving the *strong* inoculation yielded 118 colonies in a loopful of a ten-thousand-million-fold dilution at once, but after incubation for 24 hours a loopful of 1 : 10,000, 1 : 100,000 and 1 : 1,000,000 dilutions yielded 30, 1 and 0 colonies respectively; hence with *B. coli* the strong inoculation shows even a more marked disadvantage as a test method than in the case of staphylococcus, owing to spontaneous death of the organisms.

It is striking that in the case of *B. coli* as compared with *Staphylococcus aureus* the behaviour of acriflavine and proflavine seems to be reversed and the former now tends to cause the wider zone of bacteriostatic action before complete sterilisation is attained; whether this is the invariable result with the two substances we have not sufficient evidence to show, but the general result is the same with both compounds, viz. that the strong inoculation requires for practically complete sterilisation at the most 2½ times the minimum lethal concentration required by the weak inoculation.

Accordingly, great increase—many million-fold—in the number of organisms in the inoculation dose, causes merely an insignificant increase—2½–5-fold—in the concentration of the flavines necessary for practically complete sterilisation with *Staphylococcus* and *B. coli*; further, the occurrence of bacteriostatic action in much higher dilutions is a characteristic property of these substances.

We would again specially emphasise the therapeutic value of substances, like the flavines, which possess such powerful bacteriostatic properties and which are at the same time relatively non-toxic to the tissues and are not neutralised by serum. We had previously drawn attention to the ratio

$$\frac{\text{concentration of substance which inhibits phagocytosis}}{\text{concentration causing death of organisms in serum}}$$

as one means of measuring the suitability of antiseptics for acting in a wound. The significance which we attached to this *therapeutic coefficient* has been

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questioned because the time of exposure of organisms and of leucocytes to the antiseptic was not the same (Fleming), but this criticism fails altogether to take account of the fact that, short of producing actual death of the organisms, bacteriostatic action is exerted by these antiseptics. Thus, although the bacteria may still be capable of proliferating when removed into culture medium and out of contact with the antiseptic, this is of little practical importance as compared with the fact that their activities are inhibited when the antiseptic is present. Wright has also recently emphasised as an "all important principle" that the actual state of bacteria under conditions unfavourable to their activity (as where they are in contact with leucocytes in his observations or with antiseptics in our work) is not accurately shown by a method which proceeds to place the organisms under the most favourable circumstances for producing a culture.

In the case of the flavine antiseptics there exists such a wide range between the weakest concentration which will suffice to inhibit and eventually to kill organisms in a serous medium on the one hand, and on the other, the great strength which is required to paralyse phagocytosis[1] (see our previous reports), that antiseptic action and the natural defensive processes can be expected to go on side by side. No other antiseptic which has been tested, affords results which justify the belief that with it such will occur to anything approaching the same extent.

Of late there has been a tendency to question the importance of the leucocytes as a defensive mechanism in infections. Thus, prominence has been given to the fact that organisms which have been ingested by leucocytes, may be protected from the lethal action of antiseptic solutions (Jones and Rous); further, dissemination of infection has been attributed to the transport of organisms enclosed in leucocytes, and recrudescence has been explained by renewed activity of phagocytosed bacteria. The recent important experiments of Alexander, however, serve to restore perspective to the view of the anti-infective properties of the leucocytes—this worker found that virulent pneumococci when incubated along with antipneumococcus serum and leucocytes for a few hours (six), became attenuated, although they were not killed, whereas neither the antiserum nor the leucocytes by themselves produced this effect on the organisms. Thus, there is a good reason for taking into consideration the action of any proposed therapeutic agent on the activity of leucocytes when considering the properties which will determine its efficacy in the treatment of infections.

[1] Parry Morgan has also investigated the occurrence of phagocytosis *in vitro* in the presence of acriflavine and, since marked agglutination occurred in the mixture, has expressed the opinion that possibly "the phagocytosis was more apparent than real and that the organisms merely adhered to the phagocytes and were not ingested." We did not consider it necessary to refer to this point in our first report, since we had found that similar agglutination occurred with other substances, but that owing to the toxic effect of the latter phagocytosis did not take place and there were then no appearances which resembled the ingestion of organisms by the leucocytes observed in the experiments with flavine; thus *it was evident that phagocytosis occurred in the presence of flavine.*

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THE TESTING OF ANTISEPTICS OF THE CHLORINE GROUP.

Dakin and Dunham have recently observed that "in making tests of the germicidal efficiency of any antiseptic there seems to be no good reason for not following the order of mixing the materials indicated by the conditions of practical use—namely, to add the antiseptic last to the inoculated medium." With reference to our work they proceed further, "the lethal concentration of chloramine-T in serum is stated to be 1 : 250 without reference to much lower concentrations already published by others. The discrepancy is due, in the main, to the fact that the antiseptic was added first to the medium and the organisms last." We have now repeated our experiments (*a*) in the manner postulated by Dakin and Dunham, and (*b*) in the way in which they were previously performed, *i.e.* adding the antiseptic to the serum and then adding the organisms within 2 to 3 minutes afterwards, *i.e.* following Dakin's own previous publication in which this statement appears, "*Determination of Germicidal Action*....A series of tubes each containing 5 c.c. of a solution of the substance at a progressively decreasing concentration is first of all prepared, and to each tube the organism is added....The tests carried out in the presence of blood serum were performed in the same way, only the liquid in the first series of tubes contained 50 per cent. of horse serum previously heated at 55°–56° C." The results in both series are identical and confirm our previous figure (see Table V). We have been able to show that the difference between our results and Dakin's is due mainly to the fact that Dakin originally employed 50 per cent. serum—later Dakin and Dunham used 33 per cent. serum—whereas in our experiments the antiseptic mixtures contained over 80 per cent. of serum (in our experience 80 per cent. heated ox serum is a more favourable medium for the growth of staphylococci than is 40–50 per cent. serum).

The following is an example:

Medium = ox serum—previously heated at 55° C. for ½ hour—in each tube 1 c.c., (*a*) of undiluted serum, (*b*) of serum diluted with an equal volume of 0·85 per cent. NaCl solution. Chloramine-T stock solution = 1 : 23·3 (the strength of this solution was verified by titration both immediately before and after the time of employment in the experiment). Inoculation dose of organisms = 0·1 c.c. of 1 : 1000 dilution of a 24-hour peptone water culture of *Staphylococcus aureus*. In series A the organisms were added to the medium first and then the chloramine solution; in series B the chloramine was added first and then the cocci after an interval of 2–3 minutes. As a control acriflavine in undiluted ox serum was tested. Sub-cultures were made on agar after 24 hours' incubation at 37° C. The results are shown in Table V.

These results confirm our original figures, although in the present instance 20 times as many organisms were employed for the inoculation as previously. It may be noted here that to dismiss the use of small numbers of bacteria as unsuitable for testing the action of an antiseptic *in vitro*, since a similar number in a wound borders on "surgical sterility," is entirely beside the point.

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Table V.

Action of Chloramine-T on Staphylococcus aureus.

Concentration of chloramine	A: 50 per cent. serum	A: Full serum	B: 50 per cent. serum	B: Full serum
1 : 700	+	+	+	+
1 : 466	−	+	−	+
1 : 325	−	+	−	+
1 : 233	−	−	−	−

Control: concentration of acriflavine in undiluted serum, 1 : 400,000 +,
1 : 200,000 −.

+ = growth in subculture on agar.
− = no growth in subculture.

In a wound one is dealing with mechanisms inimical to the organisms (due to the leucocytes, etc.), which do not operate *in vitro*, hence the small number of organisms introduced into the culture medium is by no means subjected to the unfavourable conditions which may prevail in a wound.

VARIATIONS IN RESISTANCE OF ORGANISMS TO FLAVINES.

Acriflavine and proflavine are the most powerful antiseptics so far investigated for staphylococci and the ordinary types of *B. coli* in a serum medium. Certain strains of streptococci appear to be even more susceptible (Parry Morgan), which should be advantageous in view of the rôle of streptococci in infected wounds, although we did not find the "enterococcus" to possess more than average susceptibility. The existence of marked differences in the resistance of various species of organisms toward a particular antiseptic is now well known (see Browning). Drummond and McNee isolated from certain wounds organisms of coliform type which showed a high degree of resistance toward the flavines; we have had the opportunity of investigating three such strains and find that they all belong to a most unusual type of *B. coli*, which fails to form indol, also they are late lactose-fermenters; their culture reactions were as follows:

Motility +	glucose, lactose, saccharose, mannitol, maltose	acid and gas	dulcitol, inositol	no change	milk acid and late clot; indol negative (12 days); gelatine not liquefied

We have tested the action in serum of both acriflavine and proflavine on eleven types of *B. coli* and also on *B. pyocyaneus*, and *Urobacillus septicus*;

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the resistant strains being also included in the series; the results were as follows:

Organism	Result
B. coli (Escherich)	No visible growth in serum *plus* acriflavine or proflavine 1 : 200,000 after 48 hours at 37° C.
B. No. 71	
B. Schafferi	
B. Grünthal	
B. neapolitanus	
B. vesiculosus (2 strains)	
B. lactis aerogenes (2 strains)	
B. Morgan I	
B. paracoli type	
B. coscoroba	
B. No. 67 (inositol fermenter)	
Urobacillus septicus (*proteus* class)	

Resistant (non-indol forming) coliform B. (3 strains)—visible growth in serum *plus* antiseptic 1 : 40,000; none in 1 : 20,000.

B. pyocyaneus (2 strains): visible growth in serum *plus* antiseptic 1 : 20,000; none in 1 : 10,000.

Inoculation dose in each case 0·1 c.c. of 1 : 1000 dilution of a 24-hour peptone water culture to 1 c.c. medium.

Controls without antiseptic all yielded abundant growth, with marked turbidity of the medium.

Parry Morgan, and Bashford, Hartley and Morrison also mention resistant coliform bacilli, but give no details as to their characters. It would, therefore, be quite misleading to suggest that the typical colon bacilli of faecal origin present such resistance to flavine antiseptics as to invalidate their use generally in wounds containing coliform organisms. *B. pyocyaneus* evidently belongs to the types most resistant to flavine, which has also been observed clinically (Kellock and Harrison), but this organism appears to be relatively unimportant as a pathogenic agent in wounds. It is interesting that *B. pyocyaneus* has also been found resistant to hypochlorites (Taylor).

SUMMARY AND CONCLUSIONS.

(1) *The antiseptic and bactericidal properties of Flavines and Brilliant Green.* Extended investigations have confirmed the original values. It has been shown that the inferior potencies recorded by certain other workers depend on the use of methods unsuited for the observation of antiseptic properties, *i.e.* they fail to detect inhibition of bacterial activity *i.e.* bacteriostatic action, which is exhibited to a marked degree by flavine and brilliant green.

(2) For the therapy of a local bacterial infection, as in a wound, such bacteriostatic action is of great value. It is not essential that the chemical

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agent should by itself actually kill the organisms. Highly successful results can be obtained by a co-operation of the antiseptic and the tissues, so that the pathogenic action of the organisms is restrained. The flavines in virtue of their low toxicity to mammalian tissues and their high bacteriostatic power are therefore specially suited to act as local therapeutic agents. In addition, the fact that they are not neutralised by admixture with serum enables them to be applied clinically by a relatively simple method which does not necessitate frequent renewal.

(3) The "fundamental error" to which the method of testing chlorine antiseptics originally practised by Dakin, is liable, and which Dakin and Dunham have drawn attention to, has been shown not to affect our previous results with Chloramine-T. The difference between our values and the others is due to the fact that we employed 80 per cent. serum in the test medium, which is much more active in neutralising this antiseptic than is 33 to 50 per cent. serum employed by Dakin and his co-workers.

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