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SOME FACTORS INFLUENCING THE ACTIONS OF DYES AND ALLIED COMPOUNDS ON BACTERIA[1].

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(With Plate I and six charts.)

THE experiments recorded in this paper were undertaken in order to ascertain the effects of certain dyes and allied organic compounds on three selected species of bacteria, *Staphylococcus aureus*, *B. coli* and *B. pyocyaneus*. These experiments show that in cultures the results are influenced by many factors, and appear to suggest that the varying results of the use of these compounds in the treatment of wounds may be ascribed to some extent to the different conditions prevailing in each case.

The chief organic substances examined were diaminotrimethylacridinium chloride, termed for the sake of brevity, Homoflavine, Quinone and Crystal Violet. Homoflavine is more easily prepared on a technical scale than the ordinary acriflavine, with which it is homologous, being made from the acridine produced from metatoluylenediamine by combination with a methyl ester; both it and Crystal Violet have been produced on a manufacturing scale by Messrs Levinstein, Ltd, of Manchester, and pure material provided by this firm has been used in the present investigation. The other organic compounds named in the table on p. 4 have been carefully purified by Professor W. J. Pope; none of the dye stuffs comprised in this list were double compounds with metallic salts and their compositions are stated in Green's *Organic Colouring Matters*.

During the last few years many investigations on the bactericidal effects of dyes have been undertaken. Browning, Gulbransen, Kennaway and Thornton (1917), Dakin and Dunham (1917), and Nicholls (1917), employed 0·7 % peptone, Fleming (1917), Hewlett (1917), Morgan (1918), Taylor (1917) and Wright (1917) used "broth," Drummond and McNee (1917) "glucose broth," Morgan (1918) serum broth, Churchman (1912, 1913), Churchman and Michael (1912), Krumwiede and Pratt (1914) and Teague (1918) "ordinary agar" and Browning and Gilmour (1914) "peptone water agar." Presumably all these media contained the usual quantity of peptone. The use of peptone[2]

[1] A Report to the Medical Research Committee, April 25, 1918.

[2] Browning, Gulbransen and Thornton (1917) have shown that "for some unascertained reason the bacterial potency of flavine for staphylococcus in dilute peptone water shows considerable variations in an extended series of experiments."

has been avoided in these experiments, and consequently the results of these workers are not strictly comparable with those quoted in this paper.

Browning, Gulbransen, Kennaway and Thornton (1917), Fleming (1917) and Morgan (1918) used ox serum, Dakin and Dunham (1917) horse serum, and horse serum together with muscle extract, and Hewlett (1917) human serum. Blood was employed by Dakin and Dunham (1917), Fleming (1917) and Morgan (1918), pus by Fleming (1917) and Hewlett (1917), and milk by Hewlett (1917). None of these fluids were employed in the present experiments.

Varying results in preliminary experiments made it evident that to obtain comparable results media of simple and constant composition would be required, and that an arbitrary time limit would have to be adopted. As a fluid medium neutral meat extract and as a solid neutral meat extract agar were chosen and the actions of the compounds on the three species of organisms growing in the former and on the surface of the latter compared.

The meat extract was prepared from bullock's heart muscle after removing the fat, fascia, etc. To each 100 grms. of minced meat 250 c.c. of water were added, the fluid slowly boiled for one hour, filtered through filter paper and sterilised in the autoclave. The clear extract so obtained requires 0·08 c.c. of N/10 soda to render it neutral to neutral red.

On this medium all the organisms mentioned grow very well.

Tubes containing 1 c.c. of meat extract, 0·08 c.c. of N/10 soda, 3·5 c.c. of distilled water were prepared and sterilised by boiling. When cool 0·5 c.c. of a solution of the compound in distilled water and lastly a drop of an emulsion in sterile distilled water of the organism from a 24 hour old agar culture grown at 37° C. were added, and the culture incubated at 37° C. Care was taken to make emulsions as uniform in numbers of bacteria as possible, and on many occasions the organisms present in the cultures at the beginning of the experiment were estimated by means of plate cultures. The numbers in most of the experiments varied between 4,000 and 2,000 organisms per drop. The naked eye results were noted after 24 and 48 hours' incubation and to confirm them subcultures were sown on agar plates with a standard loop (0·01 c.c.).

In order to compare the results when the organisms were grown on the surface of agar 1 c.c. of meat extract, 2 c.c. of melted agar (2 % in distilled water), 0·08 c.c. of N/10 soda and 1·5 c.c. of distilled water were placed in tubes and sterilised by boiling. When cooled to 60° C. 0·5 c.c. of a solution of the compound was added. After thorough mixing the contents of the tube were poured into Petri dishes[1] and allowed to set. With the aid of a platinum loop three streaks of strong emulsions of the three organisms in distilled water

[1] Small Petri dishes divided according to the method devised by Churchman (1912) were employed in order to reduce the quantity of medium used and to facilitate comparison. Instead of metal divisions cardboard strips, cemented to the bottoms and sides of the dishes with water agar, were utilised.

were made across the surface of the medium. The cultures were incubated at 37° C. and the results were noted after 24 and 48 hours' incubation.

Table I (p. 4) gives the results of these experiments after 48 hours' incubation, a + indicating visible growth in the fluid medium or growth on the agar though only evidenced by the presence of a single colony, and a 0 the failure of the organism to grow.

In the agar series it frequently happened that though no growth was visible after 24 hours' incubation a few or even numerous colonies were found on examining the plate after 48 hours' incubation. Consequently the results obtained after 24 hours' incubation differ considerably from those obtained after 48 hours' incubation (Plate I, figs. 1–20). Since *in the agar series* further incubation seldom produces any change, the time limit of 48 hours was chosen, and unless otherwise stated the results recorded indicate the findings under these conditions.

In the fluid medium good growth may occur on subsequent days in tubes which show no evidence of growth in 2 days.

Table I, in which the results are tabulated in the order of the action of the compounds on staphylococci growing on the surface of neutral agar, shows that (1) under these conditions the effects of the compounds are not strictly correlated to their chemical relationship, (2) the substances most toxic to staphylococci have little toxicity to *B. coli* or *B. pyocyaneus*, and (3) of the three organisms *B. pyocyaneus* is the most resistant.

The second part of the table gives the action of the compounds on the organisms growing in meat extract, and shows that (1) under these conditions different values are obtained for many of the compounds, (2) a different order in efficiency is found, and (3) some of the compounds exhibit marked toxicity to *B. coli* and to a lesser degree to *B. pyocyaneus*.

The differences between the tables are especially noteworthy since agar has no nutritive value and the quantity of nutrient material (meat extract) is the same in both series.

Since Crystal Violet exhibits the greatest toxicity towards staphylococci, Quinone the greatest toxicity towards *B. coli* and *B. pyocyaneus*, when growing on agar, and homoflavine the greatest toxicity towards these organisms in meat extract, these three compounds were selected for further investigation.

Homoflavine.

From Table I it will be seen that on agar the toxicity of homoflavine towards staphylococci is somewhat greater than that of methylhomoflavine or methylhomoacridine, while the toxicity of these three compounds towards *B. coli* and *B. pyocyaneus* is similar. In meat extract the toxicity of all three towards staphylococci is similar, but homoflavine and methylhomoflavine are more toxic to *B. coli* and to *B. pyocyaneus* than methylhomoacridine.

Table I.

Showing the least concentrations of the following compounds which cause inhibition of growth for 48 hours at 37° C.

	Neutral agar			Neutral meat extract		
	Staphylococci	*B. coli*	*B. pyocyaneus*	Staphylococci	*B. coli*	*B. pyocyaneus*
1. Crystal violet	1:3,250,000	1:10,000	1:10,000	1:10,000,000	—	—
2. Brilliant red Rhoduline	1:1,000,000	*1: 1,000*	*1: 1,000*	1: 500,000	1: 10,000	*1: 1,000*
3. Irisamine	1: 900,000	*1: 1,000*	*1: 1,000*	1: 1,100,000	*1: 1,000*	*1: 1,000*
4. Metaphenylene Blue B	1: 200,000	*1: 1,000*	*1: 1,000*	1: 900,000	1: 10,000	1:10,000
5. Pyronine G	1: 150,000	*1: 1,000*	*1: 1,000*	1: 200,000	1: 20,000	1:10,000
6. Pararosaniline hydrochloride	1: 70,000	*1: 1,000*	*1: 1,000*	1: 60,000	*1: 1,000*	*1: 1,000*
7. Quinone	1: 70,000	1:95,000	1:30,000	1: 60,000	1:100,000	1:10,000
8. Hydroquinone	1: 60,000	1:40,000	1:10,000	1: 20,000	1: 20,000	1:10,000
9. Safranine S	1: 50,000	*1: 1,000*	*1: 1,000*	1: 70,000	1: 20,000	*1: 1,000*
10. Acridine red	1: 50,000	1:10,000	*1: 1,000*	1: 30,000	*1: 1,000*	*1: 1,000*
11. Isonitrophenol	1: 40,000	1:20,000	1:10,000	1: 20,000	1: 20,000	1:10,000
12. Chrysoïdine T	1: 20,000	1:10,000	*1: 1,000*	1: 30,000	1: 10,000	*1: 1,000*
13. Homoflavine	1: 14,000	1: 9,000	*1: 1,000*	1: 300,000	1:250,000	1:30,000
14. Methylhomoflavine	1: 10,000	1:10,000	*1: 1,000*	1: 300,000	1:250,000	1:30,000
15. Methylhomoacridine	1: 10,000	1:10,000	*1: 1,000*	1: 300,000	1:200,000	1:10,000
16. Toluidine blue acid	1: 10,000	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
17. Congo red acid	1: 10,000	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
18. Phosphine R	1: 10,000	*1: 1,000*	*1: 1,000*	—	—	—
19. Rhodamine B	1: 10,000	*1: 1,000*	*1: 1,000*	1: 10,000	*1: 1,000*	*1: 1,000*
20. Rhodamine S	1: 10,000	*1: 1,000*	*1: 1,000*	1: 70,000	*1: 1,000*	*1: 1,000*
21. Erythrosine	1: 10,000	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
22. Alizarine red	*1: 1,000*	*1: 1,000*	*1: 1,000*	1: 10,000	*1: 1,000*	*1: 1,000*
23. Orange G	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
24. Night blue basic	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
25. Diamine blue 3 B	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
26. Crystal scarlet 6 R	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
27. Benzo blue 6 B neutral	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
28. Diaminogene blue G	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
29. Quinoline yellow S	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
30. Acid violet 6 B	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
31. Magenta S	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
32. Thioflavine S	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
33. Tartrazine	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
34. Trypan red	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
35. Trypan blue	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
36. Auramine O	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
37. Patent blue	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*
38. Naphthol green	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*	*1: 1,000*

Concentrations greater than 1 : 1,000 were not tested, and the sign *1 : 1,000* indicates that good growth took place at a concentration of 1 : 1,000.

It will be noticed that the influence on staphylococci of Nos. 2 and 8 is much greater on agar than in meat extract, of numbers 10, 11, 16, 17, and 21 slightly greater, while the influence of numbers 1, 4, 13, 14, 15, and 20 is much greater in meat extract than on agar, and of numbers 3, 5, 9, 12, and 22 slightly greater. The influence of numbers 6, 7, and 19, and probably of numbers 23–38, is similar in both media.

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The effects of varying the proportion of nutrient material and the reaction of the medium.

In order to ascertain the effects of varying the proportion of the nutrient material and the reaction of the medium several series of experiments were carried out both in meat extract and on meat extract agar. In each case the total quantity of the medium employed was 5 c.c., but the quantity of nutrient material (meat extract) varied between 0·5 c.c. and 2 c.c. For example at neutrality to neutral red the meat extract series was made up in the following manner.

	Meat extract	N/10 soda	Distilled water	Solution of homoflavine
A	0·5*	0·04	3·96	0·5
B	1·0	0·08	3·42	0·5
C	1·5	0·12	2·88	0·5
D	2·0	0·16	2·34	0·5

* In this and other tables describing the composition of media the figures indicate the quantities of ingredients used in c.c.

And the agar series as follows.

	Meat extract	Agar (2%)	N/10 soda	Distilled water	Solution of homoflavine
A	0·5	2·0	0·04	1·96	0·5
B	1·0	2·0	0·08	1·42	0·5
C	1·5	2·0	0·12	0·88	0·5
D	2·0	2·0	0·16	0·34	0·5

In order to vary the reaction on the alkaline side additional quantities of N/10 soda (0·1, 0·2, 0·3, 0·4, 0·5 c.c.) were added beyond the neutral point with a corresponding diminution in the amount of water. On the acid side either no addition was made, or in some cases N/10 hydrochloric acid (0·05, 0·1 and 0·15 c.c.) was added with a corresponding diminution in the amount of water.

Thus in the experiments with the fluid medium containing 1 c.c. of meat extract the whole series was made up in the following manner.

	Meat extract	N/10 soda	N/10 HCl	Distilled water	Solution of homoflavine
I	1·0	—	0·1	3·4	0·5
II	1·0	—	0·05	3·45	0·5
III	1·0	—	—	3·5	0·5
IV	1·0	0·08	—	3·42	0·5
V	1·0	0·18	—	3·32	0·5
VI	1·0	0·28	—	3·22	0·5
VII	1·0	0·38	—	3·12	0·5
VIII	1·0	0·48	—	3·02	0·5
IX	1·0	0·58	—	2·92	0·5

The concentration of homoflavine causing complete inhibition of growth during 48 hours' incubation at 37° C. is given in Table II and Charts I and II.

Table II.

Showing the concentration of homoflavine required to cause complete inhibition of growth after 48 hours' incubation at 37° C. on agar and in meat extract of different reactions and containing different quantities of nutrient material.

Quantity of acid or alkali added	Quantity of meat extract	Agar			Meat extract		
		B. coli	Staphylococci	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*
0·1 N/10 HCl	1·0	1: 1,500	1: 4,000	1: 500	—	1: 200,000	1: 30,000
0·05 „	1·0	1: 1,500	1: 8,000	1: 500	1: 150,000	1: 250,000	1: 20,000
No addition	0·5	1: 9,000	1: 18,000	1: 500	1: 300,000	1: 500,000	1: 40,000
	1·0	1: 4,500	1: 10,000	1: 500	1: 150,000	1: 300,000	1: 20,000
	1·5	1: 3,500	1: 9,000	1: 500	1: 100,000	1: 200,000	1: 17,000
	2·0	1: 2,000	1: 6,000	1: 500	1: 100,000	1: 150,000	1: 13,000
0·08 N/10 soda	0·5	1: 11,000	1: 20,000	—			
per c.c. of meat extract*	1·0	1: 9,000	1: 14,000	—			
	1·5	1: 8,000	1: 11,000	—			
	2·0	1: 7,000	1: 10,000	—			
0·18 N/10 soda	0·5	1: 35,000	1: 30,000	—			
	1·0	1: 12,000	1: 17,000	—			
	1·5	1: 8,000	1: 15,000	—			
	2·0	1: 7,000	1: 10,000	—			
0·28 „	0·5	1: 60,000	1: 75,000	—			
	1·0	1: 20,000	1: 20,000	—			
	1·5	1: 9,000	1: 15,000	—			
	2·0	1: 8,000	1: 12,000	—			
0·38 „	0·5	1: 100,000	1: 160,000	1: 1,500	1: 1,800,000	1: 1,800,000	1: 110,000
	1·0	1: 45,000	1: 40,000	1: 1,500	1: 1,600,000	1: 1,400,000	1: 70,000
	1·5	1: 11,000	1: 16,000	1: 1,500	1: 1,100,000	1: 1,100,000	1: 50,000
	2·0	1: 10,000	1: 15,000	1: 1,500	1: 1,000,000	1: 800,000	1: 40,000
0·48 „	0·5	1: 290,000	1: 300,000	—			
	1·0	1: 90,000	1: 90,000	—			
	1·5	1: 15,000	1: 21,000	—			
	2·0	1: 14,000	1: 21,000	—			
0·58 „	0·5	1: 300,000	1: 320,000	1: 1,500	1: 2,000,000	1: 2,000,000	1: 130,000
	1·0	1: 145,000	1: 160,000	1: 1,500	1: 1,800,000	1: 1,800,000	1: 110,000
	1·5	1: 18,000	1: 23,000	1: 1,500	1: 1,400,000	1: 1,600,000	1: 100,000
	2·0	1: 17,000	1: 26,000	1: 1,500	1: 1,200,000	1: 1,400,000	1: 90,000

* 1 c.c. of meat extract requires 0·08 c.c. N/10 soda to neutralise it to neutral red. In cultures containing 0·5, 1·5 and 2·0 c.c. of meat extract proportionate quantities of N/10 soda were added. In the alkaline series 0·1, 0·2, 0·3, 0·4, 0·5 c.c. of N/10 soda were added beyond the quantities sufficient to bring the reaction to neutrality. For the sake of lucidity these figures have been omitted in Table II and Charts I, II and III, only the quantities added to cultures containing 1 c.c. being quoted.

It will be seen from Table II and Charts I and II that under all conditions the smaller the amount of nutrient material present the greater the efficiency of the homoflavine solution, and that in regard to *B. coli* and staphylococci its efficiency is greatly increased by the addition of small quantities of N/10 soda. On agar with the larger quantities of nutrient material the successive increments of soda have less effect than with the smaller quantities. In meat extract cultures this phenomenon is much less marked.

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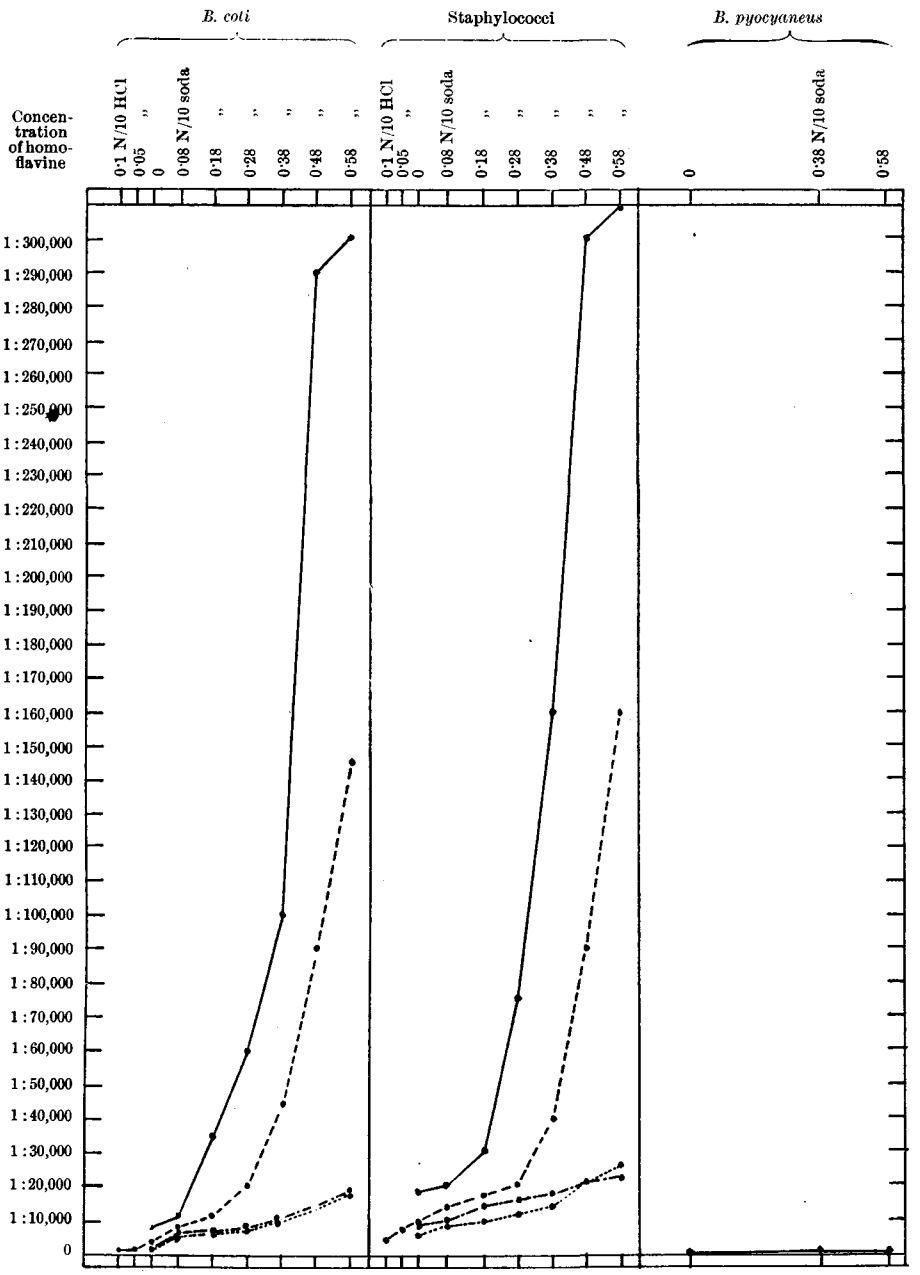

CHART I. Showing the influence of the quantity of nutrient material and change of reaction on the concentration of homoflavine necessary to cause inhibition of growth on agar during 48 hours' incubation at 37° C.

——— 0·5 c.c. meat extract —·— 1·5 c.c. meat extract
– – – – 1·0 c.c. ,, ,, ... 2·0 c.c. ,, ,,

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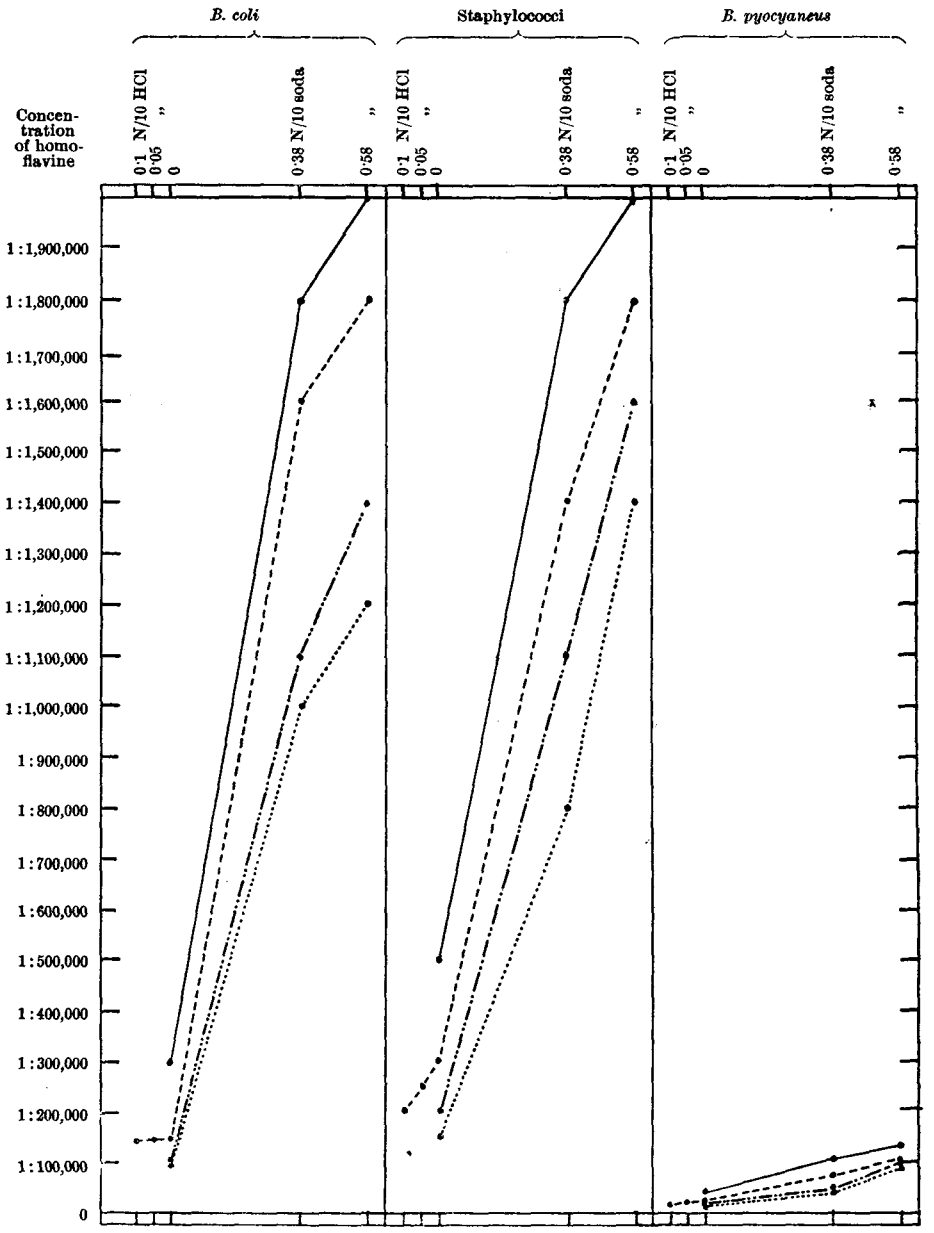

CHART II. Showing the influence of the quantity of nutrient material and change of reaction on the concentration of homoflavine necessary to cause inhibition of growth in meat extract during 48 hours' incubation at 37° C.

——— 0·5 c.c. meat extract —··—·· 1·5 c.c. meat extract
– – – – – 1·0 c.c. ,, ,, ... 2·0 c.c. ,, ,,

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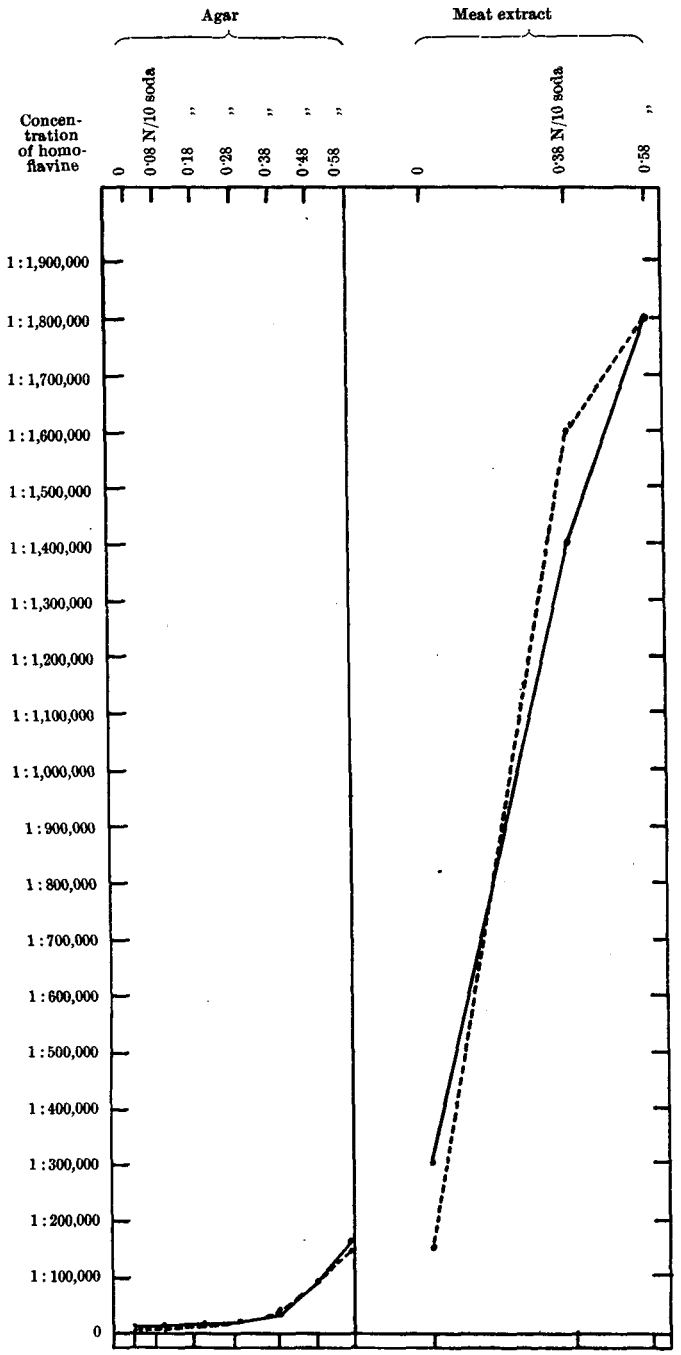

CHART III. Illustrating the differences in concentrations of homoflavine necessary to inhibit the growth of staphylococci and *B. coli* during 48 hours' incubation at 37° C. when growing on agar and in meat extract, when each tube contains 1 c.c. of nutrient material.

--- *B. coli* —— staphylococci

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It will be noticed that the curves for *B. coli* and staphylococci are very similar. In this connection it is of interest to note that these organisms multiply at much the same rate *on meat extract* (without the addition of homoflavine) as shown by counting the colonies on agar subcultures made at different periods of growth. On the other hand *B. pyocyaneus* multiplies much more rapidly.

The action of homoflavine on *B. pyocyaneus* on agar is very little influenced by the addition of soda, and not very greatly influenced in the meat extract.

Chart III has been constructed to bring out more clearly the remarkable difference between the actions of homoflavine in agar and in meat extract when acting on *B. coli* and staphylococci.

In order to ascertain whether the striking differences between the inhibiting concentrations on agar and in meat extract were due to the prevalence of aerobic conditions in the agar plates and partial anaerobic conditions in the neutral meat extract tubes, cultures were made in aerated meat extract and boiled meat extract under paraffin. The results were almost identical. The growth of the staphylococci was inhibited in each series at a concentration of 1 : 550,000, of *B. coli* at 1 : 250,000 and of *B. pyocyaneus* of about 1 : 40,000.

A series of experiments were carried out to ascertain the influence of reaction on the growth of these organisms on the media described with varying quantities of meat extract, replacing the homoflavine solution with distilled water. The results are given in the following table.

	Agar									Meat extract		
	B. coli			Staphylococci			*B. pyocyaneus*			*B. coli*	Staphylococci	*B. pyocyaneus*
	2 c.c. meat extract	1 " "	0·5 " "	2 c.c. meat extract	1 " "	0·5 " "	2 c.c. meat extract	1 " "	0·5 " "	1 c.c. meat extract	1 c.c. meat extract	1 c.c. meat extract
N/10 soda												
2·5 c.c.	−	−	−	−	−	−	−	−	−	−	−	−
2·25	−	−	−	+	−	−	−	−	−			
2·0	+	−	−	+	+	−	+	+	+			
1·9	+	−	−	+	+	−	+	+	+			
1·8	+	−	−	+	+	−	+	+	+			
1·7	+	+	−	+	+	−	+	+	+	−	+	+
1·6	+	+	+	+	+	+	+	+	+	−	+	+
1·5	+	+	+	+	+	+	+	+	+	−	+	+
1·4	+	+	+	+	+	+	+	+	+	+	+	+
N/10 HCl												
0·1 c.c.	+	+	+	+	+	+	+	+	+	+	+	+
0·125	+	+	+	+	+	+	+	+	+	+	+	+
0·150	+	+	−	+	+	−	+	+	−	+	−	+
0·175	+	+	−	+	+	−	+	−	−	−	−	−
0·2	+	−	−	+	−	−	+	−	−			
0·3	−	−	−	−	−	−	−	−	−			

+ = growth after 48 hours' incubation. − = no growth.

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This table shows that the quantity of acid or alkali which is necessary to add in order to inhibit growth depends to some extent on the quantity of nutrient material present. While on the alkaline side the range is considerable, the range on the acid side is very small, but it may be increased to some extent by increasing the concentration of meat extract. With 2·5 c.c. of meat extract growth of staphylococci occurs in the fluid medium when 0·3 c.c. of N/10 HCl is added, and with 5 c.c. of meat extract when 0·8 c.c. of N/10 HCl is present.

Such small quantities of N/10 soda as greatly influence the action of homoflavine do not of themselves appear to have any influence on the growths, when these are compared with neutral controls.

In the case of *B. coli* and *B. pyocyaneus* the organisms are very short, rounded and almost coccus-like when growing on the acid medium, and very long, thin and irregular when growing on the higher concentrations of soda.

The effect of varying the proportion of agar.

To determine whether the quantity of agar employed has any influence on the concentration of homoflavine necessary to inhibit the growth of *B. coli* and staphylococci, experiments with media of the following composition were carried out.

	Meat extract	Agar (2 %)	N/10 soda	Distilled water	Solution of homoflavine
A	1·0	1·0	0·08	2·42	0·5
B	1·0	2·0	0·08	1·42	0·5
C	1·0	3·0	0·08	0·42	0·5

After 48 hours' incubation at 37° C. it was seen that different concentrations of homoflavine were necessary to inhibit growth in these three media.

	Growth inhibited at a concentration of	
	B. coli	Staphylococci
A	1 : 14,000	1 : 30,000
B	1 : 9,000	1 : 14,000
C	1 : 7,000	1 : 9,000

Homoflavine does not seem to enter into strong combination with the agar, for if divided plates are made according to Churchman's (1912) method, having a solution of homoflavine mixed with the agar on one side and not on the other, and the partition walls are removed immediately after the agar has set, and subsequently emulsions of organisms are stroked across the medium at right angles to the dividing line, the plain medium becomes coloured for a short distance beyond the dividing line and colonies at a considerable distance beyond the line take up the stain and become yellow.

The diffusion of homoflavine and its consequences can be well illustrated in another manner. Two parallel streaks of plain agar (2 %), one broad and the other narrow, are made on the bottom of a Petri dish and allowed to set, and then medium of the composition of B in the experiment just quoted is poured into the dish so as to cover the streaks and allowed to set. Subsequently emulsions of *B. coli* and staphylococci are stroked across the plate at right angles to the agar streaks. At a concentration of 1 : 9,000 a few colonies of

B. coli grow immediately over the streaks, but not elsewhere, though when the concentration decreases to 1 : 17,000 colonies appear wherever the emulsion was spread, but most thickly over the streaks. Similarly colonies of staphylococci grow over the broad streak only at a concentration of 1 : 10,000, over both streaks at 1 : 14,000 and wherever the emulsion was spread at a concentration of 1 : 14,000 and wherever the emulsion was spread at a concentration of 1 : 20,000, showing that the homoflavine diffuses into the plain agar streaks, and consequently so lowers the concentration over them that the organisms can grow though inhibited elsewhere (Plate I, fig. 21).

Morphologically the *B. coli* from the colonies growing over the streaks at a concentration of 1 : 9,000 are very long and irregular, but those growing in the same situation at a concentration of 1 : 17,000 are much shorter and more normal in appearance. The change in morphology from very long to short normal forms may be traced in the divided plates just mentioned when passing from the extremity of the stroke of emulsion on the side containing the homoflavine to the opposite extremity.

Strong concentrations of homoflavine (1 : 1,000) seem to cause the agar to set very firmly and to lessen the exudation of water from it.

The effects of salt and of peptone on agar cultures.

In order to ascertain the influence of the presence of 0·75 % salt and 1 % peptone on agar cultures the following series A, B, C, D of experiments were carried out.

	The members of each series contained				There were added in							
					Series A	Series B		Series C		Series D		
	Meat extract	Agar	N/10 HCl	N/10 soda	Distilled water	15 % salt	Distilled water	20 % peptone	Distilled water	20 % peptone in 15 % salt	Distilled water
1.	1·0	2·0	0·4	—	1·6	0·25	1·35	0·25	1·35	0·25	1·35
2.	1·0	2·0	0·3	—	1·7	0·25	1·45	0·25	1·45	0·25	1·45
3.	1·0	2·0	0·2	—	1·8	0·25	1·55	0·25	1·55	0·25	1·55
4.	1·0	2·0	0·1	—	1·9	0·25	1·65	0·25	1·65	0·25	1·65
5.	1·0	2·0	—	—	2·0	0·25	1·75	0·25	1·75	0·25	1·75
6.	1·0	2·0	—	1·0	1·0	0·25	0·5	0·25	0·5	0·25	0·5
7.	1·0	2·0	—	1·5	0·5	0·25	0·25	0·25	0·25	0·25	0·25
8.	1·0	2·0	—	1·75	0·25	0·25	0·0	0·25	0·0	0·25	0·0
9.	1·0	2·0	—	0·2 (N)	1·8	0·25	1·55	0·25	1·55	0·25	1·55
10.	1·0	2·0	—	0·25 (N)	1·75	0·25	1·5	0·25	1·5	0·25	1·5

After 48 hours' incubation at 37° C. the results were as follows:

	B. coli Series				Staphylococci Series				*B. pyocyaneus* Series			
	A	B	C	D	A	C	B	D	A	B	C	D
1.	0	0	0	+	0	0	0	×	0	0	0	0
2.	0	0	×	+	0	0	×	+	0	0	0	0
3.	×	+	+	+	×	+	+	+	0	×	+	+
4.	+	+	+	+	+	+	+	+	+	+	+	+
5.	+	+	+	+	+	+	+	+	+	+	+	+
6.	+	+	+	+	+	+	+	+	+	+	+	+
7.	+	+	+	+	+	+	+	+	+	+	+	+
8.	+	+	+	+	+	+	+	+	+	+	+	+
9.	+	×	0	0	+	+	0	×	+	+	0	0
10.	+	0	0	0	+	+	0	0	+	0	0	0

+ = good growth, × = slight growth, and 0 = no growth.

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In the case of *B. coli* the presence of salt or peptone diminishes the range of growth on the alkaline side, and slightly increases it on the acid side, but the presence of both distinctly increases it on the acid side. In the case of staphylococcus the presence of salt has little effect, but peptone decreases the range on the alkaline side, and the presence of both increases the range on the acid side. In the case of *B. pyocyaneus* the range on the acid side is slightly increased and on the alkaline side distinctly decreased by both salt and peptone alone or combined.

The action of homoflavine on cultures of various ages.

Cultures were grown on meat extract 1 c.c., N/10 soda 0·08 c.c., water 3·5 c.c. for one, three and ten days respectively. At the expiration of these periods 0·5 c.c. of a solution of homoflavine was added, and the tubes returned to the incubator. After one, two and eight days' incubation a loopful from each tube was sown on agar, and the result recorded after two days at 37° C.

Cultures 24 hours old

Concentration of homoflavine for *B. coli* and staphylococci	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine			After 8 days' contact with homoflavine		
	B. coli	Staphylococci	Concentration for *B. pyocyaneus*	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*
1: 50,000	0	0	1: 550	0	0	0	0	few	0	0
1: 100,000	numerous	many	1: 750	0	many	0	0	numerous	0	0
1: 150,000	,,	numerous	1: 1,000	0	,,	0	0	,,	0	0
1: 200,000	,,	,,	1: 2,000	numerous	,,	0	few	,,	0	0
1: 250,000	,,	,,	1: 10,000	,,	numerous	0	numerous	,,	0	numerous

Cultures three days old

Concentration for *B. coli* and staphylococci	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine			After 8 days' contact with homoflavine		
	B. coli	Staphylococci	Concentration for *B. pyocyaneus*	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*
1: 50,000	numerous	few	1: 550	0	0	0	0	0	0	0
1: 100,000	,,	,,	1: 750	0	few	0	0	numerous	0	0
1: 150,000	,,	numerous	1: 1,000	few	,,	0	0	,,	0	0
1: 200,000	,,	,,	1: 2,000	,,	,,	0	0	,,	0	0
1: 250,000	,,	,,	1: 10,000	many	many	few	few	,,	0	0

Cultures 10 days old

Concentration for *B. coli* and staphylococci	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine		
	B. coli	Staphylococci	Concentration for *B. pyocyaneus*	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*
1: 50,000	few	0	1: 550	0	0	0	0
1: 100,000	numerous	0	1: 750	0	0	0	0
1: 150,000	,,	0	1: 1,000	numerous	few	0	0
1: 200,000	,,	few	1: 2,000	,,	,,	0	0
1: 250,000	,,	,,	1: 10,000	,,	,,	0	0

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These experiments, which have not been repeated, seem to show that in cultures during the rapidly growing stages staphylococci are killed more readily than *B. coli* by the prolonged action of certain strengths of the homoflavine. In the case of *B. coli* after a period of inhibition multiplication may occur. The older cultures of all these organisms seem to be the most susceptible to the prolonged action of homoflavine.

If cultures on such a medium (the homoflavine being replaced by distilled water) be examined by means of dilutions in plate cultures it will be found that at the end of 24 hours' incubation great multiplication of the organisms has taken place, and that by the third day the growth in the case of *B. coli* and staphylococci has nearly reached its maximum. Subsequently multiplication ceases, and by the tenth day a great reduction in numbers has taken place. *B. pyocyaneus* reaches its maximum very early, and active multiplication has ceased by the third day.

It is evident from these experiments that the results obtained by adding solutions of homoflavine to actively growing or declining cultures are very different from those obtained in previous experiments in which relatively small numbers of organisms were added to media already containing homoflavine.

The experiments which have been quoted show that the action of homoflavine is very greatly influenced by the reaction of the medium, the quantity of nutrient substance, the presence of agar, and the age of the culture; in fact any alteration in the composition of the medium, or the proportion of the ingredients, affects the results obtained to a greater or less degree. (See also pp. 16, 18).

Comparison of Acriflavine and Homoflavine.

The effects of acriflavine and homoflavine in meat extract cultures have not been compared, but some comparative experiments in ox serum, sterilised by heating to 55° C., and in 0·7 % peptone water, were made.

In each tube were placed 0·8 c.c. of serum or 0·8 c.c. of 0·7 % peptone water, 0·1 c.c. of a solution of the dye and 0·1 c.c. of a dilution (staphylococci 1 : 20,000, *B. coli* 1 : 10,000) in 0·75 % salt solution of a 24 hours' peptone water culture of the organism. Fortunately the two dilutions contained almost the same number of organisms. Control tubes without the dye were sown at the same time.

The following table (p. 15) shows the results of this experiment, the figures indicating the numbers of organisms growing in cultures on agar made with one standard loopful (0·01 c.c.), after dilution if this seemed necessary.

The peptone solution was distinctly acid, 5 c.c. requiring 0·275 N/10 soda to neutralise it to neutral red, and the serum distinctly alkaline, 5 c.c. requiring 0·75 N/10 HCl to neutralise it.

These experiments show that the actions of the two dyes on staphylococci and *B. coli* respectively are very similar, but that while the staphylococci are

Staphylococci

Concentration of dye	Cultures in 0·7% peptone: Acriflavine 5	23	51	73 hrs.	Homoflavine 5	23	51	73 hrs.	Cultures in serum: Acriflavine 5	23	51	73 hrs.	Homoflavine 5	23	51	73 hrs.
1: 50,000	–	0	0	0	–	0	0	0	–	0	0	0	–	0	0	–
1: 100,000	1	0	0	0	16	0	0	0	1	0	0	–	3	0	0	0
1: 125,000	–	0	0	0	–	0	0	0	–	0	–	*14,672	–	0	0	0
1: 150,000	6	0	0	0	16	0	0	0	20	262	7,250	365,000	1	6	1,056	1,115,000
1: 175,000	–	0	0	0	–	0	0	0	–	750	–	–	–	1	–	–
1: 200,000	7	0	0	0	14	0	0	0	33	51,000	640,000	–	16	973,000	–	–
1: 250,000	–	89,000	5,128,000	–	–	0	0	0	–	982,000	1,932,000	–	–	1,148,000	–	–
	Immediate							Immediate								
Control 30	68	12,300,000	–	–		Control 32			225	2,120,000	–	–				

B. coli

Concentration of dye	Cultures in 0·7% peptone: Acriflavine 5	23	51	73 hrs.	Homoflavine 5	23	51	73 hrs.	Cultures in serum: Acriflavine 5	23	51	73 hrs.	Homoflavine 5	23	51	73 hrs.
1: 50,000	–	0	0	–	–	0	–	0	–	0	0	0	–	0	0	0
1: 100,000	2	0	0	0	2	15	5,500	17,760	0	0	0	0	0	0	0	0
1: 125,000	–	90,880	–	–	–	50	–	39,360	–	0	–	0	–	0	0	0
1: 150,000	2	220,000	402,000	–	1	104,800	–	148,000	1	0	0	0	0	0	0	0
1: 175,000	–	400,000	–	–	–	–	–	–	–	0	0	0	–	0	0	0
1: 200,000	24	843,200	–	–	4	302,000	–	1,180,000	1	0	0	0	0	0	0	0
1: 250,000	–	1,392,000	3,208,000	–	–	–	–	–	–	0	0	0	–	0	0	0
	Immediate								Immediate							
Control 31	48	4,048,000	10,016,000	–					Control 37	427	7,112,000	–	–			

0 = no growth in subculture, – = subculture not made.

more susceptible when growing in peptone water the *B. coli* are more susceptible when growing in serum. In the lower concentrations the organisms in many cases at first seemed to decrease in numbers and then to increase.

Quinone.

Several series of experiments, comparable with those made with homoflavine, were carried out with quinone.

The effects of varying the reaction of the medium.

To test the effect of alterations in the reaction of the medium experiments were made on agar and in meat extract. In the former series the agar was melted, and the soda and water added. It was then cooled to 45° C., the solution of quinone added, the contents of the tube thoroughly mixed and the medium poured into plates and allowed to set.

	Meat extract	N/10 soda	N/10 HCl	Agar	Water	Solution of quinone
1	1·0	—	0·15	2·0	1·35	0·5
2.	1·0	—	0·1	2·0	1·4	0·5
3.	1·0	—	0·05	2·0	1·45	0·5
4.	1·0	—	—	2·0	1·5	0·5
5.	1·0	0·08	—	2·0	1·42	0·5
6.	1·0	0·18	—	2·0	1·32	0·5
7.	1·0	0·28	—	2·0	1·22	0·5
8.	1·0	0·38	—	2·0	1·12	0·5
9.	1·0	0·48	—	2·0	1·02	0·5
10.	1·0	0·58	—	2·0	0·92	0·5

The meat extract series was similar in all respects except that 2·0 c.c. of water were substituted for the 2·0 c.c. of agar. The solution of quinone was added when the medium had cooled after sterilisation.

The results are given in the following table, which shows the concentration of quinone necessary in order to completely inhibit growth for 48 hours when the agar and meat extract contain 1 c.c. of nutrient material.

	Agar			Meat extract		
	B. coli	Staphylococci	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*
N/10 HCl						
1. = 0·15	1 : 100,000	1 : 45,000	1 : 30,000	—	—	—
2. = 0·1	1 : 90,000	1 : 40,000	1 : 20,000	—	1 : 120,000	1 : 55,000
3. = 0·05	1 : 70,000	1 : 50,000	1 : 25,000	1 : 120,000	1 : 60,000	1 : 70,000
4. = 0	1 : 90,000	1 : 70,000	1 : 25,000	1 : 90,000	1 : 50,000	1 : 7,500
N/10 soda						
5. = 0·08	1 : 95,000	1 : 70,000	1 : 30,000	1 : 110,000	1 : 60,000	1 : 10,000
6. = 0·18	1 : 90,000	1 : 55,000	1 : 30,000	1 : 90,000	1 : 40,000	1 : 15,000
7. = 0·28	1 : 75,000	1 : 45,000	1 : 27,000	1 : 40,000	1 : 30,000	1 : 15,000
8. = 0·38	1 : 60,000	1 : 30,000	1 : 25,000	1 : 25,000	1 : 20,000	1 : 12,500
9. = 0·48	1 : 50,000	1 : 20,000	1 : 20,000	1 : 17,500	1 : 25,000	1 : 10,000
10. = 0·58	1 : 45,000	1 : 15,000	1 : 15,000	1 : 10,000	1 : 25,000	1 : 7,500

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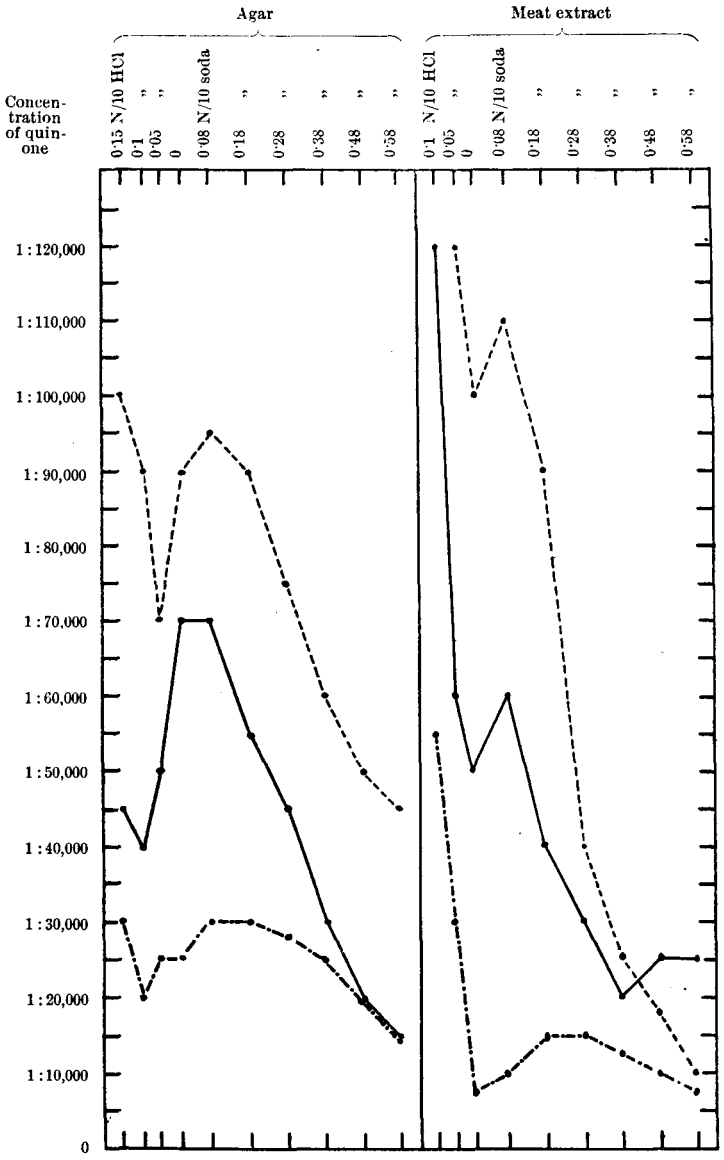

CHART IV. Showing the influence of change of reaction on the concentration of quinone necessary to cause inhibition of growth during 48 hours at 37° C. on agar and in meat extract.

---- *B. coli* —— staphylococci

-·-·- *B. pyocyaneus*

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It will be seen by comparing Charts I, II, III, and IV that while the efficiency of homoflavine increases with the increase of alkalinity the efficiency of quinone diminishes. While homoflavine is most active in an alkaline solution, quinone is most active at or near neutrality to neutral red. Further there is not so marked a difference between the concentrations of quinone necessary to produce inhibition in agar and in meat extract as there is in the case of homoflavine.

The effect of varying the proportion of nutrient material.

The efficiency of quinone decreases as the concentration of nutrient material increases as shown by the following experiment on agar.

	Composition of medium					Concentration of quinone required to inhibit		
	Meat extract	Agar	N/10 soda	Water	Solution of quinone	*B. coli*	Staphylococci	*B. pyocyaneus*
1.	0·5	2·0	0·04	2·0	0·5	1 : 150,000	1 : 110,000	1 : 15,000
2.	1·0	2·0	0·08	1·5	0·5	1 : 85,000	1 : 65,000	1 : 12,000
3.	1·5	2·0	0·12	1·4	0·5	1 : 60,000	1 : 35,000	1 : 10,000
4.	2·0	2·0	0·16	1·3	0·5	1 : 50,000	1 : 25,000	1 : 8,000

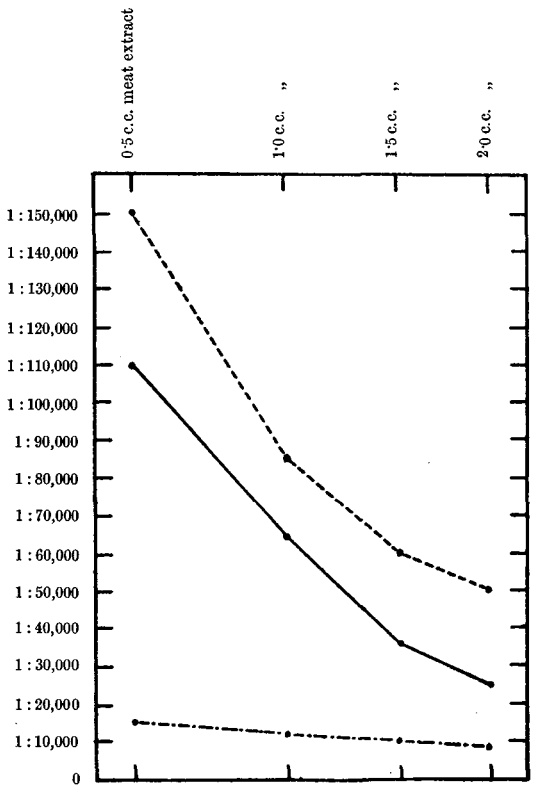

CHART V. Showing the concentrations of quinone necessary to cause inhibition of growth on agar during 48 hours at 37° C. in the presence of different quantities of nutrient material.

– – – – – – – *B. coli* ——— staphylococci

—·—·—·— *B. pyocyaneus*

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The effect of varying the proportion of agar.

The efficiency of quinone is only slightly influenced by the quantity of agar present.

	Composition of medium					Concentration of quinone required to inhibit the growth of staphylococci
	Meat extract	Agar	N/10 soda	Water	Solution of quinone	
1.	1·0	1·0	0·08	2·42	0·5	1 : 60,000
2.	1·0	2·0	0·08	1·42	0·5	1 : 70,000
3.	1·0	3·0	0·08	0·42	0·5	1 : 80,000

The effect of heat on the action of quinone.

The action of quinone, unlike the action of homoflavine, is greatly influenced by heat, as shown in the following experiments. In one series all the ingredients were mixed and the medium boiled for five minutes before pouring

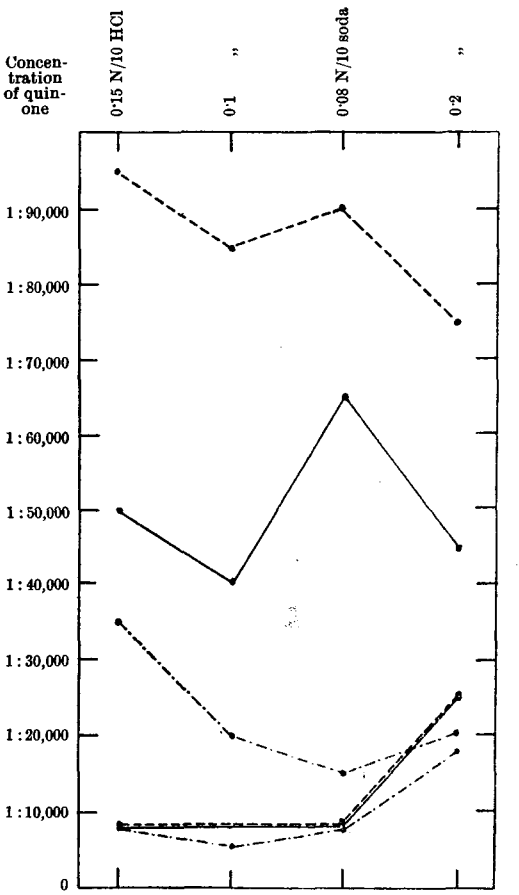

CHART VI. Showing the influence of boiling on the action of quinone. Upper lines indicate unheated series, lower lines boiled series.

------ *B. coli* —— staphylococci

—·—·— *B. pyocyaneus*

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the plates, while in the other the ingredients without the quinone were first mixed and boiled. After cooling to 45° C. the solution of quinone was added and plates immediately poured.

	Composition of media					
	Meat extract	N/10 HCl	N/10 soda	Agar	Water	Solution of quinone
A.	1·0	0·15	—	2·0	1·35	0·5
B.	1·0	0·05	—	2·0	1·45	0·5
C.	1·0	—	0·08	2·0	1·42	0·5
D.	1·0	—	0·2	2·0	1·3	0·5

	Concentration of quinone required to inhibit growth							
	Unheated quinone solution				Heated quinone solution			
	A	B	C	D	A	B	C	D
B. coli	1:95,000	1:85,000	1:90,000	1:75,000	1:7,500	1:7,500	1:7,500	1:25,000
Staph.	1:50,000	1:40,000	1:65,000	1:45,000	1:7,500	1:7,500	1:7,500	1:25,000
B. pyocyaneus	1:35,000	1:15,000	1:15,000	1:15,000	1:7,500	1:3,000	1:7,500	1:15,000

The action of quinone on cultures of various ages.

Cultures made in meat extract 1·0 c.c., N/10 soda 0·8 c.c., and water 3·5 c.c., were incubated at 37° C. for one, three and ten days respectively. At the expiration of these periods 0·5 c.c. of a solution of quinone was added to each, and the tube returned to the incubator. 24 and 72 hours later one standard loopful was sown from each culture on agar, and incubated for 48 hours.

Cultures one day old

	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
Concentration of quinone	*B. coli*	Staphylococci	Concentration of quinone	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*
1: 10,000	numerous	0	1: 1,000	0	numerous	0	0
1: 25,000	,,	numerous	1: 2,500	0	,,	numerous	0
1: 50,000	,,	,,	1: 5,000	numerous	,,	,,	numerous
1: 75,000	,,	,,	1: 7,500	,,	,,	,,	,,

Cultures three days old

	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
	B. coli.	Staphylococci	Concentration of quinone	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*
1: 10,000	numerous	0	1: 1,000	0	numerous	0	0
1: 25,000	,,	0	1: 2,500	0	,,	0	0
1: 50,000	,,	0	1: 5,000	numerous	,,	0	numerous
1: 75,000	,,	0	1: 7,500	,,	,,	numerous	,,
1:100,000	,,	0	1:10,000	,,	,,	,,	,,

Cultures ten days old

	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
	B. coli	Staphylococci	Concentration of quinone	*B. pyocyaneus*	*B. coli*	Staphylococci	*B. pyocyaneus*
1: 10,000	0	0	1: 1,000	0	numerous	0	0
1: 25,000	few	0	1: 2,500	0	,,	0	0
1: 50,000	numerous	0	1: 5,000	numerous	,,	0	numerous
1: 75,000	,,	0	1: 7,500	,,	,,	0	,,
1:100,000	,,	0	1:10,000	,,	,,	0	,,

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In the case of staphylococci it will be noticed that the older the culture the more affected it is by treatment with quinone. In the case of *B. coli* this phenomenon is not so evident. *B. pyocyaneus* is equally affected at all ages.

As in the case of homoflavine the results obtained by adding the solution of quinone to growing cultures are very different from those obtained when relatively small numbers of organisms are added to media containing a solution of quinone.

The influence of numbers of organisms and of gelatin on the effects of homoflavine and of quinone.

The influence of numbers in the initial dose of organisms.

Certain experiments illustrating the influence of variations in the numbers of the organisms in the initial dose on the effects of homoflavine and quinone were carried out simultaneously and are best considered in conjunction.

The culture medium (neutral to neutral red) consisted of meat extract 1·0 c.c., soda 0·8 c.c., water 3·42 c.c. and dye 0·5 c.c. In series A the concentration of homoflavine was 1 : 100,000 and in series B 1 : 250,000, in series C the concentration of quinone was 1 : 20,000, and in series D 1 : 50,000. Each series consisted of seven tubes to each of which one drop of an emulsion in distilled water of staphylococci from an agar culture grown at 37° C. for 24 hours was added. The first tube received a drop of a strong emulsion, the next of an emulsion of 1/10 strength, and the others of 1/100, 1/1000, 1/10,000, 1/100,000, 1/1,000,000 strengths. The organisms present in these tubes were estimated immediately by sowing standard loops (0·01 c.c.) of the cultures in melted agar and pouring plates, and in the same way the numbers were estimated, using dilutions when necessary, after 24, 48, 72 and 96 hours' cultivation at 37° C.

It will be seen that in the controls very active multiplication took place within 24 hours in all the cases tested.

In a concentration of 1 : 100,000 homoflavine, series A, a few of the organisms remained alive in tube 1, to which the greatest numbers were added, but none appeared to remain alive in the other tubes to which smaller numbers were added. At a concentration of 1 : 250,000 of homoflavine, series B, multiplication of the organisms occurred rapidly in tube 1, more slowly in tube 2, and some multiplication, after an initial diminution, in tubes 3 and 5. In the latter case the organisms were so few after 24 hours' incubation that no colonies appeared in the subculture made with 0·01 c.c. of the culture. In the other tubes no organisms appeared to survive. It seems probable therefore that, whereas in tubes 4, 6 and 7 all the organisms were destroyed in the stage when diminution in numbers is occurring, in tube 5 one or more survived, became accustomed to the conditions and subsequently multiplied.

A concentration of 1 : 20,000 of quinone, series C, inhibited growth in all cases, while with a concentration of 1 : 50,000, series D, after a great initial

diminution rapid growth occurred in tube 1, but no colonies ever appeared in subcultures from the other tubes.

No. of tube	Dilution of cocci	Diluted count per loop	Homoflavine Series A 1:100,000		Quinone Series C 1:20,000		Homoflavine Series B 1:250,000			Quinone Series D 1:50,000			
			24	48	24	48	24	48	72	96	24	48	72	96 hrs.
1.	full	83,500	57	61	0	0	752,000	4,848,000	5,424,000	5,968,000	58	5,131,000	8,352,000	8,432,000
2.	1/10	7,904	0	0	0	0	9,100	1,758,000	2,368,000	2,784,000	0	0	0	0
3.	1/100	872	0	0	0	0	29	23,300	1,536,000	2,640,000	0	0	0	0
4.	1/1,000	94	0	0	0	0	0	0	0	0	0	0	0	0
5.	1/10,000	7	0	0	0	0	0	8	10,080	2,576,000	0	0	0	0
6.	1/100,000	1	0	0	0	0	0	0	0	0	0	0	0	0
7.	1/1,000,000	0·8	0	0	0	0	0	0	0	0	0	0	0	0
							Immediate	24 hours						
Controls	1/10						7,904	6,620,000						
	1/1,000						872	5,744,000						
	1/10,000						7	5,166,000						
	1/1,000,000						0·8	2,800,000						

Several other experiments of this type gave similar results, showing that the concentration of the compound necessary to inhibit growth is greatly

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influenced by the initial number of organisms present. Small numbers of staphylococci seem to be able to overcome the influence of homoflavine more easily than the influence of quinone.

The influence of gelatin.

Several series of experiments on the influence of gelatin on the effects of homoflavine and of quinone were carried out. The first series quoted is intended to illustrate the influence of gelatin in the presence of strong concentrations of these compounds.

In this experiment small quantities of gelatin, agar or albumin were added in some of the tubes, while other tubes in the series acted as controls. All were made neutral to neutral red. The tubes were sterilised by boiling and the solution of the compound, sufficient to make a final concentration of 1 : 1,000, the albumin and a drop of an emulsion of staphylococci added after cooling.

No. of tube	Meat extract	Salt solution (3·75%)	N/10 soda	N/10 HCl	Gelatin (20%)	Agar (2%)	Egg albumin	Water	Solution of compound
1.	1·0	1·0	0·08	—	—	—	—	2·42	0·5
2.	1·0	1·0	0·17	—	1·0	—	—	1·23	0·5
3.	—	1·0	0·09	—	1·0	—	—	2·41	0·5
4.	1·0	1·0	0·08	—	—	0·5	—	1·92	0·5
5.	—	1·0	—	—	—	0·5	—	3·0	0·5
6.	—	1·0	—	—	—	—	—	3·5	0·5
7.	1·0	1·0	0·08	—	—	—	0·5	1·92	0·5
8.	—	1·0	—	—	—	—	0·5	3·0	0·5
9.	—	1·0	—	—	—	—	0·5	3·5	—
10.	—	1·0	—	—	—	—	—	4·0	—
11.	—	1·0	—	0·1	—	—	—	3·9	—
12.	—	1·0	1·0	—	—	—	—	3·0	—

Immediately after the introduction of the organisms and after 1, $5\frac{1}{2}$ and 24 hours' incubation at 37° C. standard loopfuls were sown in agar plates, the colonies on which were counted after 48 hours' incubation, with the following results.

	Homoflavine			Quinone		
Tube	1 hour	5·5 hours	24 hours	1 hour	5·5 hours	24 hours
1.	280	0	0	0	0	0
2.	3,264	168	0	0	0	0
3.	5,886	1,136	0	0	0	0
4.	488	1	0	0	0	0
5.	4,000	37	0	0	0	0
6.	2,656	360	0	0	0	0
7.	1,072	0	0	0	0	0
8.	1,696	0	0	0	0	0
	Control tubes					
	1 hour	5·5 hours	24 hours			
9.	7,200	6,016	4,448			
10.	5,984	4,624	0			
11.	26	23	2			
12.	7,616	1,632	0			

The immediate cultures yielded an average of 9950 organisms per loop.

It will be seen that a rapid diminution in the number of organisms took

place in the presence of 1 : 1,000 homoflavine in tube 1, containing meat extract only, and in tube 4, containing meat extract and agar. The numbers diminished much less speedily in tube 2 containing meat extract and gelatin, tube 3 containing gelatin only, tube 5, containing agar without meat extract, and tubes 7 and 8, containing albumin. The numbers also diminished slowly in tube 6 containing salt solution. On the other hand in a concentration of 1 : 1,000 neither gelatin, agar, albumin or salt solution seems to retard the effects of quinone.

In the control tubes 10, 11, 12, containing neutral, acid and alkaline salt solution, very few or no living organisms were found after 24 hours' incubation, but numerous colonies were present in subcultures made at that time from tube 9, containing albumin.

The influence of various quantities of gelatin, with and without meat extract, was studied in another series of experiments.

Tubes containing media of the following composition were prepared and sterilised by boiling. The solution of the compound and one drop of a staphylococcus emulsion were added when cool, and the cultures incubated at 37° C. Immediate counts showed that the cultures contained an average of 2,016 organisms per loop.

		Meat extract	Gelatin (20 %)	N/10 soda	Water	Solution of compound
Controls	1.	—	2·0	0·18	2·82	—
Controls	2.	—	1·0	0·09	3·91	—
Controls	3.	—	0·5	0·045	4·45	—
	4.	—	2·0	0·18	2·32	0·5
	5.	—	1·0	0·09	3·41	0·5
	6.	—	0·5	0·045	3·95	0·5
Controls	7.	1·0	2·0	0·26	1·74	—
Controls	8.	1·0	1·0	0·17	2·83	—
Controls	9.	1·0	0·5	0·125	3·375	—
	10.	1·0	2·0	0·26	1·24	0·5
	11.	1·0	1·0	0·17	2·33	0·5
	12.	1·0	0·5	0·125	2·875	0·5

Of numbers 4, 5, 6, 10, 11 and 12 four tubes were prepared, two (*a* and *b*) receiving solution of homoflavine of such strength as to make concentrations of 1 : 1,000 and 1 : 10,000 and two (*c* and *d*) solutions of quinone to make concentrations of 1 : 1,000 and 1 : 10,000. The organisms present in the cultures were counted by transferring standard loopfuls to melted agar and pouring plates immediately and after 24, 48, 72 and 96 hours' incubation at 37° C.

The results of these experiments are tabulated below.

	Controls		Homoflavine							Quinone						
			1 : 1,000				1 : 10,000			1 : 1,000				1 : 10,000		
No. of tube	24 hours	48 hours	No. of tube	24 hrs.	48 hrs.	72 hrs.	No. of tube	24 hours	48 hours	72 hours	No. of tube	24 hrs.	48 hrs.	72 hrs.	No. of tube	24 hrs.	48 hrs.	72 hrs.
1.	496,000	747,000	4 (*a*).	0	0	0	4 (*b*).	0	0	0	4 (*c*).	0	0	0	4 (*d*).	0	0	0
2.	424,000	596,000	5 (*a*).	0	0	0	5 (*b*).	0	0	0	5 (*c*).	0	0	0	5 (*d*).	0	0	0
3.	268,000	408,000	6 (*a*).	0	0	0	6 (*b*).	0	0	0	6 (*c*).	0	0	0	6 (*d*).	0	0	0
7.	6,416,000	10,608,000	10 (*a*).	0	0	0	10 (*b*).	60,000	1,576,000	5,680,000	10 (*c*).	0	0	0	10 (*d*).	0	0	0
8.	6,272,000	9,712,000	11 (*a*).	0	0	0	11 (*b*).	488	141	217	11 (*c*).	0	0	0	11 (*d*).	0	0	0
9.	5,582,000	7,232,000	12 (*a*).	0	0	0	12 (*b*).	3	1	0	12 (*c*).	0	0	0	12 (*d*).	0	0	0

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It will be seen that in the control tubes, 1, 2 and 3, containing gelatin without meat extract the staphylococci multiplied to a moderate extent, and the multiplication was greatest in tube 1 containing the largest amount of gelatin. Homoflavine and quinone in concentrations of 1 : 1,000 and 1 : 10,000 completely inhibited growth in gelatin alone (4 *a*, *b*, *c*, *d*, 5 *a*, *b*, *c*, *d*, and 6 *a*, *b*, *c*, *d*). In all the control tubes containing gelatin and meat extract (7, 8 and 9) great multiplication occurred, the numbers found bearing a relation to the amount of gelatin present. While homoflavine in a concentration of 1 : 1,000 completely inhibited growth in these media [tubes 10 (*a*), 11 (*a*), 12 (*a*)] multiplication occurred at a concentration of 1 : 10,000 in the tube 10 (*b*) containing the most gelatin. In tube 11 (*b*) containing less gelatin the organisms diminished in numbers but some remained alive. Almost complete inhibition occurred in tube 12 (*b*) containing the least gelatin. Quinone in both concentrations inhibited growth in all cases.

These experiments like the last show that the action of quinone is much less affected than the action of homoflavine by the presence of gelatin.

The influence of homoflavine and quinone on the liquefaction of gelatin by B. pyocyaneus.

Media of the following composition were prepared and sterilised by boiling. After cooling the solution of the compound and one drop of an emulsion of *B. pyocyaneus* in distilled water was added.

	Meat extract	Gelatin (20%) in distilled water	N/10 soda	N/10 HCl	Water	Solution of compound
A.	1·0	2·0	0·56	—	0·94	0·5
B.	1·0	2·0	0·26	—	1·24	0·5
C.	1·0	2·0	0·1	—	1·4	0·5
D.	1·0	2·0	—	0·1	1·4	0·5

The cultures were incubated at 37° C. and cooled daily in a stream of water to see if liquefaction had taken place. Subcultures made on the second day showed numerous colonies in all the tubes, indicating that, under the conditions of these experiments, 1 : 1,000 concentrations of homoflavine and quinone did not completely inhibit the growth of *B. pyocyaneus*. The results are shown in the following table (p. 26).

It will be seen that the concentrations of homoflavine used, although they do not destroy the organisms, tend to inhibit the action or the production of the liquefying ferment most strongly when the medium is alkaline, while these concentrations of quinone act in the same manner most efficiently in series C, which is nearly neutral.

Other experiments of the same kind indicated clearly that the results were correlated with the number of organisms introduced. With a large initial dose liquefaction occurred in the 1 : 2,000 concentration of homoflavine on the fourth day in series A, and in the 1 : 1,000 concentration of quinone on the third day in series D.

Homoflavine

Concentration	Series A (0·56 c.c. N/10 soda) Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14	Series B (0·26 c.c. N/10 soda) Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14	Series C (0·1 c.c. N/10 soda) Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14	Series D (0·1 c.c. N/10 HCl) Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1: 1,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 0 +	0 0 0 0 0 0 0 0 +	0 0 0 0 0 0 0 0 0 0 0 +
1: 2,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 0 +	0 0 +
1: 3,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 +	0 +
1: 4,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 +	0 +
1: 5,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 +	0 +
1: 6,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 +	0 +
1: 7,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 +	0 +
1: 8,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 +	0 +
1: 9,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 +	0 +
1: 10,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 +	0 +	0 +

Quinone

Concentration	Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1: 1,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 2,000	0 0 0 0 0 0 0 +
1: 3,000	0 0 0 +
1: 4,000	0 0 0 +
1: 5,000	0 0 0 +
1: 6,000	0 0 0 +
1: 7,000	0 0 0 +
1: 8,000	0 0 0 +
1: 10,000	0 0 0 +

Concentration	Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1: 1,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 2,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 3,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 4,000	0 0 +
1: 5,000	0 +
1: 6,000	0 +
1: 7,000	0 +
1: 8,000	0 +
1: 10,000	0 +

Concentration	Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1: 1,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 2,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 3,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 4,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 5,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 6,000	0 0 +
1: 7,000	0 0 +
1: 8,000	0 +
1: 10,000	0 +

Concentration	Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1: 1,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 2,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 3,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 4,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 5,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 6,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 7,000	0 0 0 0 0 0 0 0 0 0 0 0 0 0
1: 8,000	0 0 0 +
1: 10,000	0 0 +

Of the controls (without the homoflavine or quinone) series A liquefied on the third day, and series B, C and D became partly or completely liquefied on the second day.

+ = partial or complete liquefaction, 0 = no signs of liquefaction.

Bashford, Hartley and Morrison (1917) in recording their experience of the action of acriflavine on wounds say that "The only favourable feature has been that the patient is apparently protected in some way from the absorption of toxic products." Possibly this protection is due to the influence of the dye on ferment action as illustrated in the experiment just quoted.

Summary of experiments with homoflavine and quinone.

	Homoflavine	Quinone
1 c.c. meat extract, 3·5 c.c. water, 0·5 c.c. solution of compound.	*B. coli* inhibited at concentration of 1 : 150,000, staphylococci of 1 : 300,000, *B. pyocyaneus* of 1 : 20,000.	*B. coli* inhibited at concentration of 1 : 90,000, staphylococci of 1 : 50,000, *B. pyocyaneus* of 1 : 7,500.
Alkaline meat extract.	Efficiency greatly increased by small additions of N/10 soda.	Efficiency decreases as alkalinity increases. Most efficient at or near neutrality.
1 c.c. meat extract, 2 c.c. agar, 1·5 c.c. water, 0·5 c.c. solution of compound.	*B. coli* inhibited at a concentration of 1 : 4,500, staphylococci of 1 : 10,000, *B. pyocyaneus* of 1 : 500.	*B. coli* inhibited at a concentration of 1 : 90,000, staphylococci of 1 : 70,000, *B. pyocyaneus* of 1 : 25,000.
	Efficiency much less than in meat extract.	Efficiency nearly the same as in meat extract.
Alkaline meat extract agar.	Efficiency increases with alkalinity.	Efficiency decreases as alkalinity -increases.
Growing cultures.	Much greater concentrations required to inhibit growing cultures than organisms added to fresh media.	Action in this respect same as that of homoflavine.
	The oldest cultures are the most susceptible to prolonged action.	Action in this respect same as that of homoflavine.
Heating to 100° C.	Heating has little effect on its action.	Heating very greatly decreases the efficiency.
Initial dose of organisms.	Efficiency decreases in relation to the numbers of organisms added. With moderate numbers there is first a diminution and then a multiplication.	Relation of efficiency to numbers less marked than with homoflavine, and less tendency to multiplication after initial decrease.
Gelatin in meat extract.	Gelatin in the presence of strong concentrations of the dye retards the inhibitory action. In moderate concentrations multiplication occurs if sufficient gelatin present.	Action little affected by the presence of gelatin.
Liquefaction of gelatin by *B. pyocyaneus*.	Inhibited when medium alkaline.	Inhibited when medium neutral.
Egg albumin.	In strong concentrations of the dye retards the inhibitory action.	Action little affected.

Crystal Violet.

As shown in Table I crystal violet has relatively little action on *B. coli* or *B. pyocyaneus*, and consequently the few additional experiments carried out with this dye were made with staphylococci.

Experiments made with media of the following composition show that this dye like homoflavine acts best when the medium is slightly alkaline.

	Composition of medium						Concentration necessary to inhibit the growth of staphylococci
	Meat extract	Agar	N/10 soda	N/10 HCl	Water	Solution of dye	
A.	1·0	2·0	—	0·15	1·35	0·5	1 : 1,500,000
B.	1·0	2·0	0·08	—	1·42	0·5	1 : 3,500,000
C.	1·0	2·0	0·58	—	0·92	0·5	1 : 5,000,000

In neutral meat extract the growth of staphylococci was found to be inhibited by a concentration of 1 : 10,000,000, but the precise limits were not worked out.

Crystal violet acts more efficiently on staphylococci growing in meat extract than on ox serum.

Conclusions.

In cultures the effects of homoflavine and quinone, the two compounds most thoroughly investigated, vary on each species of organism with every change in the composition of the medium, whether the change is brought about by altering the proportion of any constituent or by the introduction of fresh constituents, and also with variations in the numbers and age of the organism. Again in each medium the concentration of the compound which inhibits each species of organism differs, and it is probable that yet other concentrations are required when mixed cultures are employed, though no experiments have been made to establish this point.

In wounds the conditions are more complex than in cultures. The conditions prevailing in no two wounds are likely to be identical, and in every wound the conditions are constantly altering, not only in regard to the chemical constituents of the fluids but also in regard to the numbers of organisms and the species and the relationships between them.

The work of Douglas, Fleming and Colebrook (1917) indicates "that bacterial symbiosis may play a very important rôle in wound infections." "Streptococci multiply much more rapidly when grown in symbiosis with various bacteria, amongst these being the group of diphtheroid bacilli which are present in practically every infected wound from the earliest stage until cicatrization is complete."

The conditions prevailing in a wound would be more closely simulated if frequent small additions of food substance were made to cultures. Under these conditions much greater concentrations of homoflavine would undoubtedly be required to cause complete inhibition of growth.

If any arguments based on results[1] in cultures of the type employed may be applied to wounds or other lesions associated with bacteria the following conclusions seem to be permissible in view of the experiments which have been quoted.

(1) No satisfactory results may be expected from the use of a dye or allied compound as a bactericidal agent unless the wound has been thoroughly cleansed before its application, since the complex organic fluids present are likely to interfere with the action of the solution.

(2) The most beneficial results are likely to be obtained if the solution of the compound is made in a fluid of the reaction at which the compound acts most efficiently, provided such a reaction is not in itself harmful to the tissues.

(3) Some compounds are more efficient than others against certain species of bacteria. In each case the dyes or other compounds used should possess special efficiency against the organisms ascertained to be present in the wound or lesion.

(4) Solutions of these compounds are most likely to produce satisfactory results if used in the very early stages of infection when the organisms are few, and unaccustomed to the new conditions in which they find themselves. Apart from killing the organisms or checking their growth the inhibitory action of certain of these compounds on some of the ferments would tend to render the conditions less favourable for bacterial growth and to hinder the production of toxic substances. When the organisms are very numerous, growing rapidly accustomed to their surroundings and protected in the fluids, these compounds are likely to be much less effective.

The last two conclusions based on culture experiments receive some support from clinical experience with acriflavine. Kellock and Harrison (1917) say that "an interesting point noticed lately has been that the antiseptic flavine appears to have no effect on *B. pyocyaneus*," and Taylor (1917) makes the following statement: "A large number of bacteriological examinations of wounds under treatment with different solutions has been recorded and instances of the specific action of certain dressing solutions demonstrated."

Drummond and McNee (1917) conclude that "flavine has many advantages as a *primary* treatment," but state that it is "not a success in the later stages." Pearson (1918) states that "in cases where infection and sepsis are active and uncontrolled the use of flavine following suitable operative measures has no beneficial effect on the subsequent progress of the case in so far as the control of sepsis is concerned."

[1] "It is generally recognized that the testing of substances for their antiseptic or germicidal properties is fraught with innumerable pitfalls and that by varying the conditions of testing almost any kind of result may be obtained" (Dakin and Dunham, 1917).

APPENDIX

CLINICAL OBSERVATIONS.

Several observers very kindly tested the action on wounds of a solution of 1 : 10,000 homoflavine and 1 : 100,000 crystal violet in distilled water. Many of these tests were carried out with the greatest care, the descriptions being accompanied by charts showing the previous treatment, the age and extent of the lesion, the rate of healing, the organisms found in smears and in cultures, etc. In some instances the progress of two similar wounds in the same individual, one treated with the solution mentioned and one with some other antiseptic, was compared. The results are described as "dramatic," "very successful," "successful," "moderate success" and "unsuccessful."

Careful though many of these inquiries have been they have not revealed the causes of the very divergent results, which occurred in the experience of several observers, and consequently it does not seem necessary to publish the cases in detail.

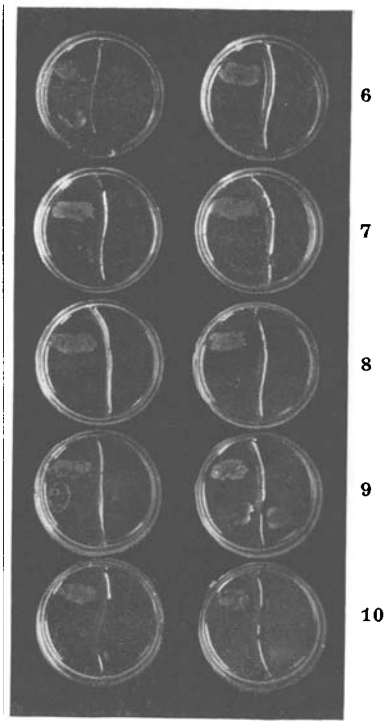
All the observations were made on relatively old wounds, and though they afford no evidence regarding the efficiency of the solution in the primary treatment of wounds, they show that old, infected wounds may be treated sometimes with "very successful" results. To ascertain the conditions under which successful results may be expected in such lesions requires combined bacteriological and chemical investigations on a series of suitable cases. Bacteriologically the species, number, rate of multiplication and symbiotic relationships of the organisms, and chemically the reactions of the fluids, the substances which inhibit the actions of the compounds, and especially the products of tissue decomposition, due to bacterial or tissue ferments, available as food material for the various species of bacteria, influence the results.

Apart from wounds this solution was employed with varying results in the treatment of gonorrhoea. Satisfactory and even "dramatic" results were obtained in several cases of stomatitis, gingivitis and pyorrhoea.

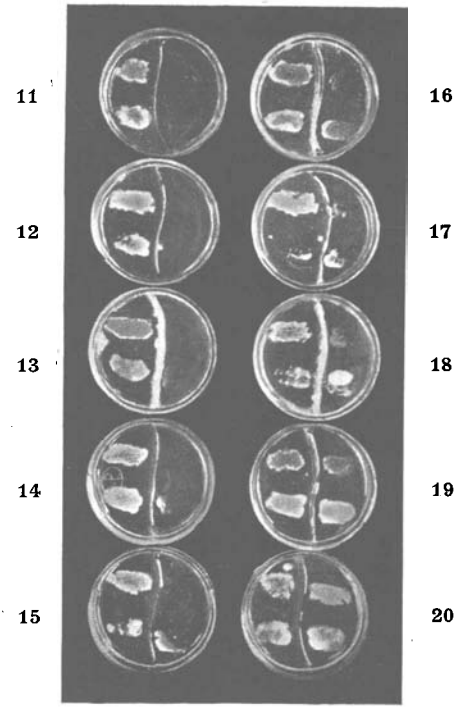
A 1 : 10,000 solution of quinone in distilled water has been tested on a small number of wounds with varying results. Like homoflavine it has given excellent results in some cases of pyorrhoea.

The three compounds differ so greatly in their powers of inhibiting the growth of such organisms as *B. coli*, staphylococci and *B. pyocyaneus* under varying cultural conditions that it might be desirable to test the action on wounds of a solution in distilled water of 1 : 10,000 homoflavine, 1 : 10,000 quinone and 1 : 100,000 crystal violet.

Pharmacologically these compounds are very inert, as shown by experiments which Professor A. R. Cushny, F.R.S., Dr W. E. Dixon, F.R.S., and Dr D. Cow very kindly carried out.



Figs. 1—10



Figs. 11—20

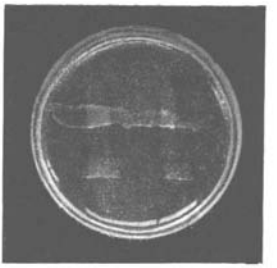


Fig. 21

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EXPLANATION OF PLATE.

Figs. 1–20. Photographs to show the differences of growth on agar after 24 hours' and after 48 hours' incubation at 37° C.

Figs. 1–10 represent Petri dishes divided according to Churchman's (1912) method after 24 hours' incubation at 37° C. The left side in each case is the control, and contains a medium composed of agar 2 c.c., ox pancreas extract[1] 0·5 c.c., distilled water 1·0 c.c., and N/10 soda 0·5 c.c. The right side contains a medium composed of agar 2 c.c., ox pancreas extract 0·5 c.c., distilled water 0·6 c.c., N/10 soda 0·5 c c., and the solution of homoflavine 0·4 c.c. The concentration of homoflavine in dish 1 was 1 : 100,000, in dish 2 1 : 200,000 and so on up to 1 : 1,000,000 in dish 10.

An emulsion of *B. coli* in distilled water was streaked across the upper part of each side of each dish and an emulsion of staphylococcus across the lower part of each side.

Figs. 1–10 show the growth of *B. coli* in each case on the control side, but in most cases no visible growth of the staphylococcus. On the right side no visible growth of either organism has occurred in 24 hours at 37° C.

Figs. 11–20 represent the same dishes after 48 hours' incubation. On the left or control sides considerable growths of both *B. coli* and staphylococcus are seen. On the right sides a slight growth of staphylococcus is seen first in fig. 14 with a concentration of 1 : 400,000 homoflavine, and it becomes more marked in the later dishes, being almost equal to the controls for fig. 16 (homoflavine 1 : 600,000) onwards. Growth of *B. coli* is seen first in fig. 16 with a concentration of 1 : 600,000 homoflavine. In concentration of 1 : 900,000 and 1 : 1,000,000 (figs. 19, 20) the growths are equal to the controls.

These photographs indicate sufficiently the necessity for adopting a rigid time limit in recording the results of such experiments.

Fig. 21. A Petri dish on the bottom of which two vertical streaks of plain agar were made (seen as light coloured vertical bands), and allowed to set. Over them was poured a medium composed of meat extract 1 c.c., agar 2 c.c., N/10 soda 0·08 c.c., distilled water 1·42 c.c. and 0·5 c.c. of a solution of homoflavine, making a final concentration of 1 : 17,000.

An emulsion of *B. coli* was streaked transversely across the upper part of the dish, and the colonies of this organism are seen stretching across the dish. Across the lower part of the dish an emulsion of staphylococcus was streaked. It will be noticed that the colonies of this organism are growing only over the streaks of plain agar.

The photograph represents the condition after 48 hours' incubation at 37° C.

[1] Numerous experiments with pancreas and other organ extracts were made, but have not been quoted, as new factors are introduced which have not been worked out sufficiently.

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