CHEMOTHERAPEUTIC EQUILIBRIA

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The statement has been made (1), with reference to the apparent fruitlessness of the search for specific chemotherapeutic agents, that "the real difficulty lies in the necessarily opportunistic experimental method, and the lack of a rational scientific means of approach." The considerations presented in the present paper are sufficiently general to be valid regardless of the mechanism of chemotherapeutic action which may be eventually discovered; and it is hoped that they will suggest a tentative rational approach to such a study.

Bacteriochemical Equilibria

A large amount of work done *in vitro* has indicated that the reaction between the bacterial cell and some, at least, of the common chemotherapeutic agents is chemical in nature and indicates a behavior which can be predicted by the law of mass action (2).

The bacterium is pictured as an equilibrated amphoteric system of more than one component. Such systems have been shown to behave in many respects as simple ampholytes (3).

The belief that the reaction between the organism and the agents studied is chemical is founded on extensive studies of staining behavior, bacteriostatic behavior, and the effect of oxidizing agents and decolorizing agents on the former. The following series of equilibria may be thought of as establishing themselves in a system of bacteria and basic agent. For considerations of staining actions this agent will be a basic dye; for bacteriostatic considerations it may or may not be a dye. We will indicate the fact that the agent is basic by the type formula DOH, and that the bacterium is amphoteric by the formula HBOH.

1. $DOH \leftharpoons D^+ + OH^-$
2. a) $HBOH \rightleftharpoons H^+ + BOH^-$
   b) $HBOH \leftharpoons HB^+ + OH^-$
3. $D^+ + BOH^- \rightleftharpoons DBOH$
4. $DBOH + HOH \leftharpoons HBOH + D^+ + OH^-$

Maximum retention of stain, or maximum bacteriostatic effect, will be expected under conditions favoring maximum formation of the unionized dye-bacterial compound, DBOH (Equation 3). The longer arrows in the above set of equations indicate the directions in which the equilibria would shift due to an increase in alkalinity.*

For staining reactions we have the following predictions and verifications:

1. Increase in pH should increase the retaining power for dye. This fact is now generally recognized. At sufficiently high pH values even Gram negative organisms may appear to be Gram positive (2c).

2. The acidic strength of the organism should be a factor. Actually, determinations of the isoelectric ranges of a number of organisms showed that these ranges in the case of Gram positive organisms occurred at a significantly higher acidity than those characteristic of the Gram negative organisms as a class (2f) (4).

3. Any process which tends to increase the acidic strength of the same organism should increase its inherent retaining power for dye. Actually it has been shown that by oxidation the retaining power of any organism for basic dye is increased. By sufficiently vigorous oxidation any Gram negative organism can be made to appear Gram positive. Subsequent reduction tends to reverse this effect (2c).

4. If these reactions represent the partial attainment of an equilibrium condition, they should be easily reversible. Actually this is demonstrated by the ease of decolorizing any non-capsulated organism with an acid decolorizer.

5. If the type of bond between organism and dye is chemical, then the general nature of the decolorizer used should determine its functioning as a decolorizer apart from the mere solubility of the dye in it. In this connection the authors have shown (2j) (2k) that basic decolorizers show abnormally high decolorizing power toward smears stained with acid dyes, and acid decolorizers show the same abnormal behavior toward smears stained with basic dye. It should be borne in mind that this effect is independent of any pH effect on decolorization and depends only on the chemical nature of the decolorizers. For example aniline would be a representative basic decolorizer while aldehydes would be acidic in nature.

In the case of acid dyes what may be thought of as a reverse behavior to that toward basic dyes has been in every case found to exist. Such could be predicted from an analogous set of equations.

Analogously we would have for bacteriostatic reactions, the following predictions and verifications:

1. Increase in alkalinity, which shifts the equilibria toward the formation of com-

* Equation 4 takes account of the fact that the effect of increasing alkalinity on the amphoteric cell is much greater than on the purely basic dye through the ordinary pH range where such studies are of importance, even though the respective ionization constants may not be so widely different. In this pH range the dyes, at least, would be almost wholly in the form of salts and thus, for comparison of effect only, the dye is represented as being highly ionized compared to the protein constituent.

pounds represented by the type formula DBOH in the equations, should increase the effective dilution of a basic bacteriostat. In the case of dyes this is, as is now well known, the case where such increase in alkalinity is not sufficient to precipitate out the agent or to alter its chemical nature (2g) (2h).

2. These bacteriostatic effects should also be reversible. That is, as an example, a concentration of a dye which would just inhibit growth at a certain pH should fail to inhibit it at a lower pH. This has been definitely shown by the authors (2g). Such a reversal can also be brought about in other ways. Any method of dissociating the molecule represented by DBOH by removing the basic D^+ ion will be effective. This is shown by the work of Englehardt (5), who removed mercuric ion by sulfide precipitation, and found that staphylococci which had been treated for 72 hours in a 1% bichloride solution would, after the removal of the mercuric ion, grow out. Similarly apropos in this connection are the results obtained by Churchman (6) on injection of stained *Bacillus anthracis*, which were found to remain apparently innocuous for periods from 10 to 20 times longer than those required by unstained organisms to produce death, but which finally suddenly revived with fatal results. Here, of course, we cannot put our finger on the chemical mechanism by which the positive dye ion was removed from action.

3. We should expect increase in basic strength of dye to increase its effectiveness. As the authors have pointed out (2e), all available data indicate such to be the case, but such data are meager. This factor cannot be generally treated because different classes of agents show effects which are of an entirely different order of magnitude. Also through the pH range usually studied most of them will be in salt form and but slightly hydrolyzed.

4. The presence of protein or other matter of a similar nature which chemically resembles bacteria should decrease the effectiveness of any agent by binding a portion of it in an ineffective combination. This has been shown to be so by Graham-Smith (7), Winslow and Dolloff (8), Wels (9), and others.

5. If mass action tends to govern bacteriostatic effects, the amount of bacterial inoculum should be a factor. That this is so has been qualitatively demonstrated by many, and semi-quantitatively shown by Gay and Beckwith (10), Browning and Gulbransen (11) and Graham-Smith (7), among others. While this last mentioned phenomenon has received other explanations, it is at least completely in harmony with the point of view outlined above.

6. Finally we may expect the acidic strength of the bacteria to be a factor. The well known fact that basic dyes are more effective against Gram positive than against Gram negative organisms, coupled with our findings on the isoelectric ranges of these organisms showing the Gram positive ones to be more strongly acidic, constitutes the experimental verification of this prediction.

The probability of the general correctness of the above outlined point of view is strengthened by the analogous behavior of organisms toward acidic substances, which behavior can also be successfully predicted from an analogous set of equations, as well as by the general similarity of all such behavior to the simple protein chemistry.

Stoichiometrical Relationships

Whatever be the mechanism of internal therapy, there seems to be evidence *in vitro* of some action between agents and organism which follows ordinary stoichiometrical laws. It is therefore of interest and importance, in the first place, to inquire whether the quantities of therapeutic agent which have been suggested from time to time are stoichiometrically sufficient. Recently the dyes gentian violet and mercurochrome have been used to a considerable extent in blood therapy. Take the former as an example.

A concentration of 1:10,000 in the blood stream has been found safe and has been used. Taking the equivalent weight of the dye as about 400, we have, at this

TABLE I

Showing the Effect of Blood Constituents on the Effective Concentration of Dye against Bacteria in the Blood

Equiv. dye ion orig.	Approx. equiv. protein anion	Approx. ionization const. for dye-protein salt*	Equiv. dye ion. final	Equiv. bacterial protein	Approx. ratio $\frac{\text{Dye equiv.}}{\text{Bact. equiv.}}$
1	20 (plasma)	0.0005	0.01	0.00004	250.
1	35 (whole blood)	0.0005	0.006	0.00004	150.

* This magnitude is obtained from experiments of the type described in section on "Abnormal ionization equilibria."

concentration, one equivalent of dye to about 4,000,000 parts of blood. In the blood we have, besides the bacteria which are being combated, the blood proteins to consider, especially those of the plasma which, at the pH of blood, are on the alkaline side of their isoelectric points. These proteins are in large excess compared with the bacteria. If we assume that they have characteristics not too dissimilar from those simple vegetable proteins so exhaustively investigated by Hoffman and Gortner (12) we can get a fair idea of the order of magnitude of the concentration of protein anion. In Table I are recorded results of such calculations. We have assumed the composition of the blood as given by Mathews (13). A preliminary determination of the ionization constant of the dye base of gentian violet indicated that, since it is added as the chloride, the dye will be present practically entirely as cation until bound by bacteria or some component of the blood. The number of equivalents of fibrinogen can be assumed by reading directly from the curves of Hoffman and Gortner for fibrin; while the number of equivalents of the other proteins are assumed to be about the same as they would be if they consisted of the

prolamines studied by these workers, twelve of which showed almost identical behavior at blood pH. The various quantities in Table I are calculated on the basis of one equivalent of dye ion, i.e. for 4,000,000 parts of blood.

The bacterial equivalents are calculated on the assumption of a count of 10,000 per cc. (2e). The final ratios, then, represent lower limiting conditions. Work on the staining behavior of blood cells, both sheep and human (15) indicates that the cells as a whole have an isoelectric point at a pH of about 6 to 6.4 so that at blood pH they are just beginning to show an appreciable affinity for basic dye. It is therefore more than probable that they do not affect the effective dye concentration to nearly the extent assumed in the values given in Table I. Moreover a bacterial count of 10,000 per cc. seems to be also a fairly limiting case. Under actual conditions the effective dye-bacterial ratio would probably be nearer many hundred than the values given.

These values indicate that, even in blood where it might be expected that dye would be completely combined with blood proteins which are somewhat in excess, a total dye concentration found clinically safe to use, 1:10,000, may be expected to furnish a bacteriostatic value corresponding to an *in vitro* concentration of 1:1,000,000 or 1:2,000,000 (i.e. 0.01 to 0.006 parts in 10,000 as in column 4 of Table I). These latter concentrations are not only in large stoichiometric excess over bacterial equivalents but are concentrations found bacteriostatic *in vitro* for many strains, especially of Gram positive organisms.

Recently some work reported by Hirschfelder and Wright (14) indicates that a concentration of crystal violet 1:20,000 in the presence of 1% albumin showed the same antiseptic power as a 1:35,000 solution in the absence of protein. No mention is made of buffering the system though the results were obtained by a process in which CO_2 was liberated. This, in an unbuffered system, might significantly affect the pH, which has been shown (2g) to greatly influence the effectiveness of dyes, and one cannot judge critically the conclusions of the authors that even adsorbed dye has some antiseptic value. This conclusion was reached on the basis of calculations from adsorption data which indicated that this 1:20,000 dye solution should have had the same antiseptic power in 1% albumin as a 1:125,000 solution in water. Details of measuring adsorption are not given so that one cannot judge the applicability of these results to blood conditions, but the work would tend to confirm the conclusions reached from the above calculations.

Time of Establishing Dye-Protein Equilibrium

A puzzling feature regarding the action of therapeutic agents, especially in the blood, which has often been brought up, is the apparent rapid disappearance of the agent. In this connection the results of two experiments are presented.

Table II embodies the results of some time experiments on the establishment of practical equilibrium between finely cut dry gelatin slabs and solutions of gentian

violet. Naturally the time values may be in error by 50% or more on such short times, but the results are suggestive. In obtaining these results definite amounts of gelatin were shaken vigorously with definite volumes of gentian violet solution of known concentration and controlled alkalinity, for definite periods of time. A portion of the dye was by this means bound by the gelatin and removed from

TABLE II

Showing the Approximate Length of Time Required for Equilibrium between Gentian Violet and Gelatin

cc. Dye soln.	Grams gelatin	cc. N/2 NaOH added	Time shaken (sec.)	Orig. dye concn. p.p.m.	Final dye concn. p.p.m.	Final dye concn. p.p.m. (blank)
10	0.08	—	60	10	8.0	9.0
10	0.08	0.20	10	10	5.0	
10	0.08	0.35	10	10	2.0	7.5
10	0.10	0.20	10	10	5.0	9.5
	(same tube)		25		3.0	
			40		3.0	
			60		3.0	8.5

TABLE IIa

10	0.10	—	20	10	7.0	
10	0.10	0.04	12	10	6.0	
10	0.10	0.20	10	10	4.0	9.0
10	0.10	0.40	10	10	3.5	9.0
10	0.10	—	20	5	3.5	
10	0.10	0.04	12	5	3.0	
10	0.10	0.20	10	5	1.5	
10	0.10	0.40	10	5	1.0	4.5

(Longer shaking in these latter cases made no difference in the results.)

Results in Table II were obtained using a solution of gentian violet in water; those in Table IIa were obtained using a solution of the dye in M/20 disodium phosphate.

solution, and the intensity of the color remaining in the supernatant liquid was compared with standard solutions of the dye.

This experiment indicates that, if the above pictured mechanism of bacteriostasis is true, the time required for a bacteriostat to become effective is conditioned largely by speed of mixing with an infected fluid and perhaps of penetration into organisms. The actual bacteriostatic equilibrium seems to establish itself very rapidly when mutual contact of dye with the particular component with which it may combine is obtained.

This simple experiment indicates that, when there are no disturbing factors and when agitation is vigorous, a primary equilibrium is very rapidly reached between protein and dye. Although, in the case of blood therapy, the method of administration of the agent would seem to lead to a rather rapid and thorough mixing, one may expect in such a medium, where blood proteins are so enormously in excess of bacterial protein, that the time required by the agent for action may be considerably increased.

It should be pointed out that the time required for the establishment of this type of equilibrium should not be confused with the times, reported through the literature, required for "killing" an organism. An agent in the blood may be effectively holding in check the normal development of an organism, yet when a sample of this same blood is plated out it may appear by no means sterile. This may be due as we have seen above to an alteration of any of the factors which have been shown to reverse a bacteriostatic equilibrium.

Alteration of Therapeutic Agent in the Blood

The other consideration we wish to present in connection with the apparent rapid disappearance of such an agent from the blood is that this apparent disappearance may not mean that the blood no longer retains some effect. It seems to be necessary for certain agents, notably certain pentavalent arsenic preparations, to be reduced by the body tissues to trivalent arsenic before they produce the desired effect. It was on such a basis that Ehrlich accounted for the clinical trypanocidal effectiveness of atoxyl, which, *in vitro*, is ineffective against these organisms even at a concentration of 5 per cent. On the other hand the trivalent arsenic in the form of the oxide is immediately trypanocidal at a concentration of 1:100,000.

Gentian violet is rather easily decolorized with either nascent hydrogen or hydrogen peroxide. The following experiments were performed.

Solutions of the colorless products were prepared using both methods. For the first method zinc and hydrochloric acid were employed, and the bulk of the zinc was removed by precipitation with ammonia. The resulting solution was tested for bacteriostatic action. Controls of ordinary gentian violet as well as of the zinc salt, prepared from zinc and acid as in the reduction mixture, were run. The bac-

teriostatic activity of a dye solution decolorized by hydrogen peroxide and in which the excess peroxide had been decomposed was also tested. Results are presented in Table III. They are, of course, preliminary in nature, but they seem to have a significance in themselves aside from their bearing on a rational picturization of a mechanism for blood therapy, especially in the case of agents which seem to rapidly disappear. It may be pointed out that reduction by zinc and acid does not

TABLE IIIa

Showing the Bacteriostatic Effect of Decolorized Gentian Violet

Organism	pH	Zn soln. control	G. V. red. by nascent hydrogen sample #1	G. V. red. by nascent hydrogen sample #2	Normal G. V. control	Dilution of agent
B. coli.....	5.5	+ + +	− − −	− − −	− − −	1:20,000
Strain 1	6.5	+ + +	− − −	− − −	− − −	1:20,000
B. coli.....	5.5	+ + +	− − −	− − −	− − −	1:20,000
Strain 2	6.5	+ + +	− − −	− − −	− − −	1:20,000

TABLE IIIb

Organism	Dilution of agent	pH	G. V. red. by peroxide	G. V. red. by nascent hydrogen	Normal G. V control
B. coli.....	1:10,000	5.5 to 6.0	− −	− −	− −
B. coli.....	1:20,000	5.5 to 6.0	− +	+ +	− +

TABLE IIIc

B. coli.....	1:10,000	5.5 to 6.0	− −	− −	− −
B. coli.....	1:20,000	5.5 to 6.0	− −	− −	− −

give the colorless product quite a fair chance in this test, since the gelatinous zinc hydroxide, which comes down in considerable quantities, is bound to carry down with it a considerable amount of active material which is thus removed.

The medium was ordinarily plain nutrient broth adjusted to a certain pH and containing a known amount of bacteriostat. In Table IIIa are given growth results in two samples of the dye decolorized with nascent hydrogen for 24, 48 and 72 hour incubation periods. (In this particular experiment the medium was 1%

lactose broth.) In Tables IIIb and IIIc results are given for growth in one sample of dye decolorized by nascent hydrogen and in one decolorized by hydrogen peroxide. Results are for 24 and 48 hour incubation periods. A slow precipitation of zinc hydroxide, brought about by pH adjustment, seemed to carry down nutrient material and, presumably, active agent as well. Therefore a trial was made on tubes in which the precipitation was hastened as much as possible and the precipitate centrifuged out. 24 and 48 hour growth results are given in Table IIIc, otherwise this table is similar to IIIb.

We are not, of course, suggesting that gentian violet is decolorized by these mechanisms in the blood, but it is of interest to note that disappearance of color in the case of such a reagent does not in itself mean disappearance of therapeutic effect.

At first sight these results may seem in disagreement with the statement of Dubos (16) who, from a study of the bacteriostatic effect of certain dyes which form reversible oxidation-reduction systems, finds that "the dyes are not toxic in the reduced form." His results are not comparable with those reported here, however, since he is working with reversible systems, systems which are reduced by the medium rapidly even in concentrations as high as about 1:5,000 and therefore systems which would tend to poise the medium at a certain oxidation potential. This poising effect he points out as a factor in the mechanism of dye bacteriostasis. There is no doubt in the minds of the present authors as to the validity of this claim where it can be shown that the bacteriostatic systems are effective as poising agents. In this paper bases for the claim of another factor are given. The relative importance of the two factors will depend on the particular systems. The most direct evidence of this other factor, which may be thought of as covalent salt formation between bacteriostatic agent and some constituent of the organism, will be found below.

Flocculation Equilibria

It may seem at first sight a far cry from the above specifically chemical equilibria to the phenomenon of flocculation, with its possible relation to agglutination, which we usually consider as a surface phenomenon of physical nature. Results given in Table IV are typical. From such results as these it is indicated that flocculation behavior is influenced by essentially the same factors and in essentially the same way as is staining behavior.

Using care to differentiate between so-called acid agglutination and flocculation produced by the agent under observation, the following facts were ascertained:

TABLE IV

Showing the Effect of Dye Concentration and of pH on the Time of Flocculation of the Gram Negative B. coli and the Gram Positive B. cereus by Means of Basic and of Acid Dye

Floc. time (min.)	Basic fuchsin pH (approx.) 1	2	3	4	5	6	Floc. time (min.)	Acid fuchsin pH (approx.) 1	2	3	4	5	6
Organism—*B. coli*													
Dye concentration 1:200													
1	−	−	−	−	+	+	1	±	±	−	−	−	−
3	−	−	±	+	+	+	5	±	±	−	−	−	−
4	−	±	±	+	+	+	8	+	±	−	−	−	−
17	±	+	+	+	+	+	44	+	+	±	−	−	−
Dye concentration 1:250													
1	−	−	−	−	±	±	7	±	±	−	−	−	−
3	−	−	−	±	+	+	42	+	±	±	−	−	−
7	−	−	±	+	+	+							
22	±	+	+	+	+	+							
Organism—*B. cereus*													
Dye concentration 1:200													
0.5	−	−	−	−	+	+	2	±	±	−	−	−	−
2	−	−	+	+	+	+	11	+	+	±	−	−	−
3	−	±	+	+	+	+	19	+	+	±	−	−	−
4	±	+	+	+	+	+	60	+	+	+	±	−	−
Dye concentration 1:250													
1.0	−	−	−	−	±	+	1	±	±	−	−	−	−
1.5	−	−	−	±	+	+	7	+	±	−	−	−	−
2.0	−	−	±	+	+	+	17	+	±	±	−	−	−
2.5	−	±	+	+	+	+	60	+	+	±	−	−	−
6.0	+	+	+	+	+	+							

1. The basic dyes, gentian violet and fuchsin, are more effective in flocculating both the Gram negative *B. coli* and the Gram positive

B. cereus at higher than at lower pH values. That is, at constant dye concentration the flocculation is more rapid, while to attain a certain flocculation speed less dye is necessary, at high than at low pH values.

2. The reverse is true in the case of acid fuchsin. It should be pointed out in connection with material presented under "Abnormal ionization equilibria" that neither brucine nor nicotine cause flocculation at concentrations comparable to those of the dyes mentioned above. The experiments were made repeatedly, checking the macroscopic readings by microscopic examination.

If we picture the cell membrane, as suggested by Bayliss (17), as a variable structure in equilibrium with the changing states of the cell, the above results are easily brought into harmony with the general picture.

Any material within the cell which lowers the surface energy will accumulate at the surface. It has been shown (18) (19) (20) that alteration of the surface tension of media through wide ranges has no apparent effect on the viability of many bacteria. This means that the bacterial cell possesses a mechanism for adjusting its surface tension in response to changes in the surface tension of the medium so as to bring about a fairly constant interfacial tension between its surface and the medium. The obvious mechanism is a labile distribution equilibrium, between the surface and the interior of the cell, of some surface tension depressant such as a lipin or even a protein constituent. Addition of a reagent which possesses the power to bind such a substance will of course shift any surface equilibrium with the result that the interfacial tension between organism and medium may increase. The tendency therefore will be to decrease total surface and flocculation will result. Such binding agents are the dyes in the above experiments, their binding power having been previously shown to vary with pH in the manner necessary to explain the flocculation results.

It may be suggested that change in pH and addition of dyes may alter the surface tension of the medium in a way to cause flocculation. Measurements of surface tension of nutrient broth and of broth containing as high as 1 per cent dye and with pH varying between 2.5 and 8 gave a variation of only about 5 dynes, however, so that some other explanation must be sought than this.

Abnormal Ionization Equilibria

The question may well arise why, if we imagine the action of therapeutic agents to be pictured as taking place according to the set of equilibria formulated above, there is such a wide difference between the inhibiting dilutions of different groups of agents which seem to belong to the same general chemical type. More specifically the question may be put why such substances as the triphenylmethane dyes, for example, inhibit bacterial growth at effective concentrations enormously less than those at which other basic substances, whose basic strength may be as great or greater, are found to be effective. This question has led one of the authors to an investigation of equilibria of the type represented by equation number 3 above, namely the ionization of the compound represented by the formula DBOH. Obviously if this compound is highly ionized enormous excess quantities of D^+ ion would be necessary for it to be formed in appreciable amount.

To determine whether different types of basic substances behave in a significantly different manner with regard to the ionization of their protein salts, conductivity measurements were made on solutions of certain bases alone, on a solution of protein alone, and on mixtures of the two. Results are given in Table V.

For the protein a 1% gelatin solution adjusted to a given pH by means of NaOH was used. For the bases, potassium, as the chloride, nicotine as the free base and gentian violet as the chloride were employed at concentrations equivalent to that of the gelatin taking its equivalent weight toward bases as 3300 (21).

In the cases of both nicotine and potassium the measured conductivity of the mixture agrees with that calculated on the assumption that none of the ionic species present tend to form undissociated molecules. In the case of the dye-protein system, however, there is a significant "loss of conductivity" when the two are mixed. A considerable portion of the dye or protein ion disappears, and this can be most easily pictured as being due to the fact that we are here dealing with a salt type of compound which is only slightly ionized, analogous in its behaviour to such inorganic salts as lead acetate, mercury salts, and others, which, compared with the general run of inorganic salts, are only slightly ionized.

The results of this experiment offer an explanation for the difference in flocculation behavior between the alkaloids and dyes noted in the above section. In the same way they offer an explanation for the

findings (22) that nicotine, even at a concentration of 1:2,000, does not seem to inhibit the growth of either Gram positive or Gram nega-

TABLE V

Showing the Effect of Mixing Solutions of Protein Anion with Various Cations on the Conductivities of These Ions

Solution	Temp.	pH	Conductivity x 10^6 (measured)	Conductivity x 10^6 (calculated)	% Difference
A. Ionization of potassium proteinate					
KCl	23.5	unbuffered	108.6		
gelatin	23.5	7.65	38.4		
1:1 mixture	23.5	7.6	75.1	73.5	2.
B. Ionization of nicotine proteinate					
nicotine	24.9	9.5	13.5		
gelatin	24.9	9.4	39.0		
1:1 mixture	24.9	9.35	27.1	26.3	3.
C. Ionization of gentian violet proteinate					
gentian violet	23.6	7.25	27.0		
gelatin	23.6	7.3	38.4		
1:1 mixture	23.6	7.1	23.0	32.7	−30.
1 gel: 2 dye	23.6	7.15	18.5	34.6	−46.

The values in column 4, Table V, are the conductivities of the ions under consideration and not the total measured conductivities. KCl is corrected for the Cl ion, gelatin is corrected for the conductivity of the Na ion added in pH adjustment, etc. These corrections do not in any way affect the argument, though they do affect the magnitudes in the last column. If we take the total conductivities we find that for nicotine and potassium the observed conductivities for the mixture do not differ from the calculated values by more than a fraction of 1%. The decrease in the case of the dye in this way amounts to 13 to 18%. Since this decrease is not distributed among all of the ionic species present in the mixture but is practically confined to the ones discussed, a more definite idea of its magnitude can be had from the corrected data as given. The last system, namely gelatin and dye, has received fairly extensive study and results in detail are to be published elsewhere.

tive organisms. The dye in each case actually seems to bind, in unionized combination, some protein-like constituent of the bacterial

cell, in the one case shifting the surface equilibrium and causing flocculation, and in the other case interfering with normal cell development; whereas the alkaloid, not seeming to form such a type of linkage, is ineffective in both cases.

Ionic Displacement Reactions

It may be suggested that the above conductivity results are explicable on the basis of the mutual flocculation of oppositely charged colloidal particles and has no connection with any type of salt formation. In answer to such a suggestion the following experimental results are presented.

TABLE VI

Showing the Liberation of H-Ion When Gelatin Is Mixed with Dye Cation, and of OH-Ion When It Is Mixed with Dye Anion

	Crystal violet		Acid fuchsin	
	pH	$-\Delta$(pH)	pH	Δ(pH)
dye	3.92		3.13	
gelatin	3.91		3.13	
1:1 mixture	3.83	0.08	3.32	0.19
dye	4.64		3.75	
gelatin	4.64		3.75	
1:1 mixture	4.29	0.35	4.10	0.35
dye	5.64		4.60	
gelatin	5.64		4.62	
1:1 mixture	5.32	0.32	4.73	0.11

Consider an amphoteric substance represented by the type formula HBOH, which can exist in solution not only in the form of the neutral compound but also either as cation or anion depending on pH. Let it react with a basic dye ion and there are the following possibilities:

$$D^+ + BOH^- = DBOH$$

and

$$D^+ + HBOH = DBOH + H^+$$

If these equations represent the types of reaction taking place between dye and protein, there ought to be conditions, if the compound DBOH is sufficiently stable, such that H-ion is liberated when dye ion and protein in unionized form are mixed.

In an analogous manner there ought to be conditions under which, when an acid dye ion is substituted for the basic dye ion, OH-ion should be liberated. This can be easily tested out by adjusting dye solutions and protein to the same pH and noting any change on mixing (23). A large number of such experiments have been carried out using various proteins and various dyes. Typical results are given in Table VI, using gelatin as a protein, crystal violet as a basic dye and acid fuchsin as an acid dye. The gelatin was a 1% solution and the crystal violet was used as a solution of 1.5 grams per liter. The other dye solution contained 3 grams per liter.

Here the basic dye causes a decrease in pH corresponding to a liberation of H-ion, and the acid dye causes the opposite effect.

Is it possible that types of therapeutic specificity will be found to be somehow connected with the property of forming such un-ionized compounds with a cell constituent? This is at least a point worthy of consideration.

SUMMARY

1. The general adequacy of the bacteriostatic mechanism for the action of dyes which postulates a mass law equilibrium between bacteriostat and organism, which latter is pictured chemically as an ampholyte, is discussed.

2. It is shown that, even in blood, where, with safe concentrations of dye, there seems to be a significant excess of protein over dye, the stoichiometric excess of dye required by the above mechanism is available.

3. Experiments are presented indicating that the time required for such a bacteriostat to act is very short, being probably conditioned largely by speed of mixing or of penetration.

4. Apparent disappearance of dye from blood stream need not mean that the blood has lost bacteriostatic value.

5. Data are presented indicating that the behavior of dyes in causing flocculation of organisms is affected by the same factors and in the same way as in inhibiting growth.

6. Direct evidence of ionic combination between dye ion and protein ion is presented by noting conductivity decrease when the two ions are mixed, and also noting the displacement of H-ion from un-ionized protein by dye cation, or of OH-ion by dye anion.

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