IONIZATION, pH AND BIOLOGICAL ACTIVITY

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1. GENERAL CONSIDERATIONS

Biological activity is known to be influenced by many physical and chemical factors; of these the degree of ionization is recognized as outstandingly significant. A change in biological response has often been observed to accompany a change in the degree of ionization of a drug, or of its biological receptor, or of both. These changes in ionization are brought about (a) by changing the pH of the medium surrounding a receptor (this may affect both receptor† and drug) or

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† *receptor:* Molecule, or part of a molecule, playing an important part in the metabolism of an organism and capable of combining with foreign molecules (see Section 6).

(b) by making a small change in the molecular structure of a drug so as to modify its tendency to ionize (4).

A high proportion of commonly used drugs are capable of ionization somewhere within the physiologically interesting pH range. Some of these, like sodium chloride, remain completely ionized within this range, but the great majority ionize to different degrees as the pH is varied.

The variable ionization of drugs would not be a matter of great interest to pharmacologists if both an ion and its non-ionized counterpart (*i.e.*, molecule) had the same biological action. However, in all cases which have been investigated, the ion and its corresponding molecule behave differently, so that the control of ionization is seen to be essential in biological experiments if they are to yield their full content of meaning. In some cases, the difference in action between an ion and its molecule partakes of an all-or-nothing character.

2. THE CHEMISTRY OF IONIZATION

Many substances are known which do not increase the electrical conductivity of water when dissolved in it. These are called non-electrolytes (chloroform and ether are examples) and they depress the freezing-point of water proportionally to their molar concentration. Acids, bases and salts, on the other hand, all increase the electrical conductivity of water when dissolved in it. These are called electrolytes, and the majority of drugs are electrolytes. All electrolytes depress the freezing-point of water to a greater extent than would have been expected from their molar concentration. In the cases of hydrochloric acid, sodium hydroxide and sodium chloride this depression is double what would have been expected and this led the Swedish chemist Arrhenius to formulate the theory of ionization of electrolytes (1884–1887), which has been brought up-to-date by Debye and others. Thus, in solution, hydrochloric acid consists entirely of hydrogen kations and chloride anions ($H^{\oplus}$ and $Cl^{\ominus}$), sodium hydroxide of sodium kations and hydroxyl anions ($Na^{\oplus}$ and $OH^{\ominus}$) and sodium chloride of sodium kations and chloride anions ($Na^{\oplus}$ and $Cl^{\ominus}$). Sodium sulphate gives three times the expected depression, and it has been shown that instead of each expected molecule of Na_2SO_4, three ions are present, namely, two sodium kations and one sulphate anion ($SO_4^{\ominus\ominus}$).

(i) *The Complete Ionization of Salts*

In general, salts are completely ionized in solution (11, 17), but there are a few exceptions, the halides of mercury, cadmium and lead being the most notable. Bi- and tri-valent metals form complexes with phosphoric acid and with polyvalent organic acids (such as citric acid or glycine), but these complexes are not true salts and have little tendency to ionize (see section 7, below).

Not only are the strongest solutions of salts completely ionized, but fused salts are also (32, 41). Moreover, X-ray analyses of a salt, for example, sodium chloride crystals, show that no *molecules* of NaCl exist, the chloride and sodium ions being evenly spaced throughout the crystal, as in solution. Thus, the old conception that salts could, under some circumstances, exist as molecules is seen

to be unfounded. It is true that the ions in crystals and fused solids are not hydrated as in solution and hence are somewhat different in properties, for example, they are poorer conductors of electric current. In strong solutions (*e.g.*, above 10 per cent), there is a pronounced tendency for oppositely charged ions to cluster together as in crystals, an effect which becomes more pronounced when both ions have valencies higher than one. Thus magnesium sulphate is *dissociated* to the extent of only 50 per cent in decinormal solution, notwithstanding the fact that it is completely *ionized* at this and all other concentrations; complete dissociation is achieved on further dilution.

Because of the great dilutions which he commonly uses, it is seldom necessary for a biologist to consult tables (23, 28) of *thermodynamic activity coefficients* which have been drawn up for the conversion of *ionic concentrations* into *ionic activities* (which are the concentrations of solitary, fully hydrated ions). Apart from the exceptional halides of mercury, cadmium and lead (mentioned above), it is safe to take activities of inorganic salts as equal to concentrations at the dilutions commonly used. Organic salts may be treated in the same way, except where one of the ions is large (*i.e.*, with a formula-weight of over 200; see Section 7 (iv)).

Because salts are completely ionized, they have no biological properties other than those of the individual ions of which they are composed. Thus calcium chloride can have no conceivable physiological effects other than those peculiar to calcium ions and to chloride ions. This simple conception needs modification when a salt is derived from either a weak acid or a weak base, because more or less of the non-ionized acid or base will be liberated by hydrolysis (see (ii), below) thus adding its own biological effect to those of the constituent ions of the salt. We shall see (vi, below) how this hydrolysis can often be overcome by a small change in pH.

That the physiological properties of a salt should be no more nor less than the sum of those of its ions is often overlooked by biological workers. For example, Hata (37) examined the toxicity of 2:8-diamino-10-methylacridinium chloride and iodide for the mouse, and found the iodide half as active, weight for weight. He then compared the ability of these substances to save the lives of mice infected with streptococci; once again the iodide was half as active. Now, as both of the anions are biologically inert at the great dilutions tested, the biological activities must be proportional to the amount of kations in these substances (which are completely ionized in neutral solutions (3)). The formula-weights are respectively 260 and 351, so that the iodide should have 74 per cent of the potency of the chloride. The biological results are in as good agreement with this calculation as could reasonably be expected for serial dilutions. However, the author gave no indication that he realized that his results were inevitable, for he had only been comparing the diaminomethylacridinium kation with itself.

The antibacterial properties of a number of other acridine salts have been tested as their chlorides, sulphates, nitrates and iodides (12), and no effect attributable to the acid radical was found, beyond experimental error. This was only

to be expected. However, in 1944, two authors published a statement that the hydrochloride of "Atebrin" is 32 times as bacteriostatic as the corresponding lactate against haemolytic streptococci, and in the next year they stated that the formate was four times as active as the tartrate against *Clostridium welchii*. These observations were published without the comment for which they obviously call. Now, it is conceivable that "Atebrin", because of its high formula-weight of 400, is showing thermodynamic activity effects and that these would vary with different anions. However, this could not be the explanation in the present case because far less striking differences were seen when these salts were tested on other organisms. This makes it likely that the salts which appeared to be less potent were not completely in solution in the relevant tests (undissolved material does not usually enter into rapid equilibrium with the surrounding fluid).

(ii) *The Ionization of Acids and Bases*

Unlike salts, acids and bases need not be completely ionized in solution. Strong acids (*e.g.*, hydrochloric acid) and strong bases (*e.g.*, sodium hydroxide) are completely ionized in the pH range of 0–14, but many biologically-active substances are weak acids or bases and hence show variable ionization within this pH range. Even small variations in pH on either side of the neutral point (pH 7) make considerable changes in the proportions of drug ionized in such cases as barbiturates, alkaloids, local anaesthetics and anti-histaminics. Several examples will be given later.

Salts which are formed from a weak acid or from a weak base are not stable in solution, but hydrolyse partly to the acids and bases from which they are derived. In this way a salt (which is by definition completely ionized) can give rise to an acid or base which is incompletely ionized. This situation is not as confusing as it may seem because the degree of ionization in solution depends on only two factors, the pH and the pK_a. The latter (which will be defined in (iv) below) is a constant for any acid or base. Hence, if the pH is controlled, the degree of ionization depends only on the nature of the acid (or base) added, *regardless of whether or not it has previously been neutralized*. Thus, the same ratio of atropine ions to atropine molecules will result from the addition of atropine hydrochloride, atropine sulphate or free atropine to a bath that has been buffered at pH 7. If the pH of the bath is raised, the proportion of atropine ions to atropine molecules will decrease, but the new ratio will again be independent of the form in which the atropine was added.

Not long ago, a paper was read at a scientific congress in which the author investigated the comparative effect of boric acid and one of its sodium salts (borax) on blowfly larvae. Had the substances been used as such, some physiological difference might have been observed because boric acid is acidic and borax is alkaline. However, the author had, very properly, carried out his experiments in a buffered solution with the result that no significant chemical difference existed between his two solutions. He actually found that both substances gave the same quantitative response. Although this puzzled him at the time it does

show that the biological technique was sound because the experiment actually tested nothing else.

In 1944, the bacteriologists whose work with "Atebrin" has been referred to (under (i), above) published another paper in which they found that 5-aminoacridine was 64 times more bactericidal than its hydrochloride (against pneumococcus type III in glucose-broth). The authors did not say whether their medium was buffered. If it was buffered, the same result should have been obtained in both cases and hence the technique was at fault. If the medium was not buffered, the results could not have quantitative significance because of the differences in pH between solutions of a strong base and of its salts (the striking effect of pH changes on the antibacterial action of acridines has been known since 1919 (13, 31)).

(iii) *The Ionization Constant (K_a)*

An essential part of Arrhenius's theory of ionization was the application of the *law of mass action* to describe the state of ionic equilibrium. Thus, acetic acid (CH_3COOH) is a weak acid which ionizes in water to give some hydrogen ions ($H^{\oplus}$) and some acetate anions ($CH_3COO^{\ominus}$). The product of the concentration of the ions (which is $[H^{\oplus}]\,[CH_3COO^{\ominus}]$ always bears a fixed ratio to the concentration of the unaffected molecules $[CH_3COOH]$. This ratio is called the acidic ionization constant (K_a), or more simply the ionization constant. Thus:

$$K_a = \frac{[H^+][CH_3COO^-]}{[CH_3COOH]} \qquad \text{(i)}$$

and this has been found, experimentally, to be 1.75×10^{-5} (at 25°).

Sometimes the expression "dissociation constant" is used for ionization constant, but the latter is more precise. Many complexes, such as enzyme systems, "dissociate" into their components, and the relevant equilibria can be expressed by dissociation constants similarly derived from the law of mass action. Nevertheless, such constants are not usually ionization constants.

The state of ionization of weak bases also can be described by acidic ionization constants. For example, ammonia is a weak base which can take up hydrogen ions to form ammonium ions. This is, of course, equivalent to thinking of the ammonium ion ($NH_4^{\oplus}$) as a weak acid which ionizes in water to give some hydrogen ions ($H^{\oplus}$) and some molecules of ammonia (NH_3). Thus:

$$K_a = \frac{[H^+][NH_3]}{[NH_4^+]} \qquad \text{(ii)}$$

and this has been found, experimentally, to be 5.5×10^{-8} (at 25°).

The use of acidic constants to describe the ionization of bases was introduced in 1923 by Brönsted (10) who noted the formal similarity between equations (i) and (ii). It is most advantageous to have the ionization of both bases and acids expressed on the same scale, but earlier workers wrote,

$$K_b = \frac{[OH^-][NH_4^+]}{[NH_4OH]} \qquad \text{(iii)}$$

and the value of K_b (the basic dissociation constant) was found to be 1.8×10^{-5} (at 25°). Equation (iii) is of little use because it does not touch the heart of the matter which is this: an acid produces hydrogen ions and a base receives them. Thus both acid and base can be related in terms of a single quantity, their affinity for the hydrogen ion. This relationship requires the use of the acidic ionization constant for both acids and bases.

(iv) *The Definition of pK_a*

Ionization constants are small and inconvenient, but their negative logarithms (known as pK_a values) are convenient to speak and to write. Thus the pK_a of acetic acid is 4.76 and of ammonia, 9.26. The older literature often gives pK_b values for bases (*e.g.*, 4.74 for ammonia); these can be converted to pK_a values by subtraction from the negative logarithm of the ionic product of water (K_W) at the temperature of determination. The value of pK_W is 14.16 at 20°, 14.00 at 25° and 13.58 at 37°.

It is evident that pK_a values provide a very convenient way of comparing the strengths of acids (or of bases). The stronger an acid is, the lower its pK_a; the stronger a base is, the higher its pK_a.

An acid or base when half ionized has a pH equal to its pK_a. When an acid is 10 per cent ionized (or a base is 90 per cent ionized), the pH is 1 unit below the pK_a. When an acid is 90 per cent ionized (or a base is 10 per cent ionized), the pH is 1 unit above the pK_a. Any acid or base is an effective buffer within the range from one unit below to one unit above the pK_a value. Biologists can therefore use initiative in selecting buffers suitable for particular experiments. The range in the literature is unnecessarily restricted; it neglects kations, and uses many anions (*e.g.*, citrate and phosphate) which form nonionized complexes with the ions of essential trace metals. Thus, phosphate buffer inhibits the action of *iso*citric dehydrogenase in the pig heart by removing manganese (42).

(v) *Tables of Useful pK_a Values*

The common pK_a values may be obtained from standard textbooks (*e.g.*, 9). Others are easily located through *Chemical Abstracts* where they are indexed under "Ionization, electrolytic." Table 1 gives the relative strengths of some common acids and bases; this table is intended to be committed to memory. Acids and bases of equivalent strength will be found opposite one another.

A value of 4-5 (*c.f.* acetic acid) is typical of a great many monocarboxylic acids, both aliphatic and aromatic. The value of 10 for phenol is typical also of its homologues (*e.g.*, thymol and the cresols). Acids with pK_a values much greater than 7 scarcely redden litmus.

The value of 11 for ethylamine is typical of aliphatic bases; that of 5 for aniline is typical of aromatic bases which are much weaker. As pK_a figures are logarithms, there is a difference of one million-fold (*i.e.*, antilog 6) between the strengths of ethylamine and aniline.

The presence of electron-attracting groups (*e.g.*, —Cl or $—NO_2$) weakens bases and strengthens acids, usually by 1 or 2 pK_a units; but when the group is suitably placed, the effect can be made much greater (*c.f.* aniline and *p*-nitroaniline in

Table 1; phenol and trinitrophenol (picric acid, pKa 0.8); acetic acid and trichloroacetic acid (pK_a 0.7)). It is also possible for the chemist to strengthen bases and weaken acids to almost any desired extent.

A selection of polybasic acids is given in Table 2. It is interesting to note how the values for citric acid lie close together, whereas those for phosphoric acid are widely spaced.

The pK_a values of several naturally occurring bases will be found in Table 3. The values for alkaloids were determined by Kolthoff in 1925 (40). Acetylcholine and curarine do not appear to have been investigated but probably resemble other quaternary bases (*e.g.*, tribenzylmethylammonium chloride, which is 13.4 at 18°). Guanidine (pK_a 13.7 at 25°) is a strong organic base of another type, being strengthened by a resonance effect which favours the ion at the expense of the molecule.

TABLE 1

Approximate strengths of some common acids and bases

ACIDS	pK_a	BASES	pK_a
Hydrochloric acid	*	Sodium hydroxide	*
Oxalaic acid	2		12
	3	Ethylamine	11
	4		10
Acetic acid	5	Ammonia	9
Carbonic acid	6	Quinine, Strychnine	8
	7		7
	8		6
Hydrocyanic acid, Boric acid	9	Aniline, Pyridine	5
Phenol	10		4
	11		3
	12		2
Glucose	13	*p*-Nitroaniline	1

* Too strong to have a pK_a value.

(vi) *Calculation of the Percentage Ionized*

The percentage ionization of any acid or base can be calculated if two things are known, (a) its pK_a value and (b) the pH at which the information is required. For a base, equation (iv) is used.

$$\text{Per cent ionized} = \frac{100}{1 + \text{antilog}\,(\text{pH} - \text{p}K_a)} \qquad \text{(iv)}$$

and for an acid, equation (v) is required

$$\text{Per cent ionized} = \frac{100}{1 + \text{antilog}\,(\text{p}K_a - \text{pH})} \qquad \text{(v)}$$

These equations show that the degree of ionization varies with the pH. The relationship between ionization and pH is not linear, but sigmoid as Fig. 1 clearly shows. From this figure, it is evident that a small change in pH can make a large change in the percentage ionized, particularly if the values of pK_a and pH lie

close together. For example, if pilocarpine (pK_a = 7.3) is being used at pH 7.6 it is only 33 per cent ionized; but if used at pH 7.0, it is 67 per cent ionized.

TABLE 2

pK_a *values of some polybasic acids (20°C)*

ACID	ANION	pK_a
Carbonic	mono-	6.46
	di-	10.35
Citric	mono-	3.06
	di-	4.74
	tri-	6.41
Oxalic	mono-	1.19
	di-	4.29
Phosphoric	mono-	2.16
	di-	7.13
	tri-	12.30
Sulphuric	mono-	*
	di-	1.93
Tartaric	mono-	3.01
	di-	4.55

* Too strong to have a pK_a value.

TABLE 3

pK_a *values of some naturally-occurring bases (15°–20°C)*

BASE	pK_a	BASE	pK_a
Adrenaline		Morphine	
kation	10.2	kation	8.2
anion	8.7	anion	9.9
Arecoline	7.5	Nicotine	8.2
Atropine	10.0	Papaverine	6.3
Cocaine	8.7	Pilocarpine	7.3
Codeine	8.3	Piperidine	11.6
Emetine		Quinine	
mono-kation	8.6	mono-kation	8.4
di-kation	7.7	di-kation	4.7
Eserine	8.2	Quinidine	8.9
Ethanolamine	9.5	Strychnine	8.4
Gelsemine	9.4	Tyramine	
Histamine		kation	9.3
mono-kation (NH_2)	9.9	anion	10.9
di-kation	6.1		

Thus a variation of only 0.6 unit in the pH has doubled the concentration of kations and halved the concentration of molecules. Table 4 gives an overall picture of the changes in ionization produced by variation in (pK_a − pH), and

Table 5 provides a magnified picture of this effect in the critical region where pK_a and pH are nearly equal.

3. EXAMPLES OF SUBSTANCES WHOSE CHEMICAL REACTIVITY DEPENDS UPON THE DEGREE OF IONIZATION

The organic chemist often has to use strong acid or alkali to facilitate a synthesis. Reactions which require such reagents for their initiation have been shown to depend on the ionization of a difficultly ionizable substance (34). Dimethylaniline (I) provides an interesting example. This substance ordinarily nitrates in the *o*- and *p*-positions, as shown by the arrows. However, if sufficient

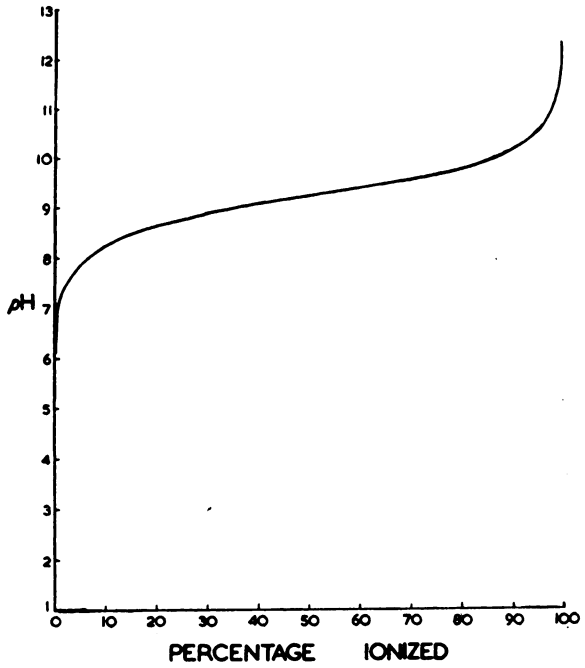
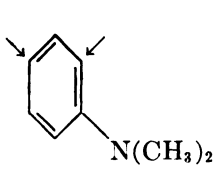
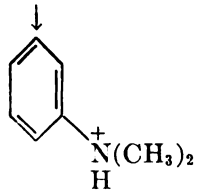


FIG. 1. Typical curve obtained in the potentiometric titration of an acid (boric acid, pK: = 9.21 at 20°).

concentrated sulphuric acid is present for the substance to be entirely converted to the kation (II), nitration occurs exclusively in the *m*-position. The hydrolysis of sucrose, when catalysed by dilute acids, is actually the hydrolysis of the small amount of sucronium kation which the acid continually forms (34).



(I)



(II)

Quite obviously the action of drugs is not concerned with ionizations occurring so far from the neutral point as these examples; a better model is supplied by ascorbic acid. This substance in alkaline solution is readily oxidized by air to give

TABLE 4*

Calculation of the extent of ionization, given pK_a *and* pH

pK_a − pH	PER CENT IONIZED (IF ANION)	PER CENT IONIZED (IF KATION)
−4	99.99	0.01
−3	99.90	0.10
−2	99.01	0.99
−1	90.91	9.09
0	50.00	50.00
1	9.09	90.91
2	0.99	99.01
3	0.10	99.90
4	0.01	99.99

* An extended form of this Table is available (4).

TABLE 5

Calculation of the extent of ionization where pK_a *is close to* pH

pK_a − pH	PER CENT IONIZED (IF ANION)	PER CENT IONIZED (IF KATION)
−0.9	88.81	11.19
−0.8	86.30	13.70
−0.7	83.37	16.63
−0.6	79.93	20.07
−0.5	75.97	24.03
−0.4	71.53	28.47
−0.3	66.61	33.39
−0.2	61.32	38.68
−0.1	55.73	44.27
0	50.00	50.00
+0.1	44.27	55.73
+0.2	38.68	61.32
+0.3	33.39	66.61
+0.4	28.47	71.53
+0.5	24.03	75.97
+0.6	20.07	79.93
+0.7	16.63	83.37
+0.8	13.70	86.30
+0.9	11.19	88.81

dehydroascorbic acid. Now, ascorbic acid is capable of giving three different ionic species, the ratios of which vary according to the pH, in the usual way. These three species are the di-anion, the mono-anion and the molecule. As Table 6 (top half) shows, decreasing the alkalinity slows the rate of oxidation in

proportion as the concentration of di-anion is lowered, even though the proportions of the other two species are thereby increased. In the presence of cupric ions, the autoxidation of ascorbic acid goes much faster; but the mechanism is obviously different, for here the rate is proportional not to the concentrations of the di-anion or of the molecule, but to that of the mono-anion (Table 6, lower half).

Malonic acid is readily decarboxylated to acetic acid when in the molecular condition; however, the mono-anion decarboxylates at only one tenth this rate and the di-anion does not decarboxylate at all (33).

4. HOW IONIZATION CAN INFLUENCE BIOLOGICAL ACTION

(i) *Ionic Bonds and Covalent Bonds*

The ready reversibility of drug action by dialysis, which has so often been demonstrated, points to the rarity of covalent bonds being concerned in drug-

TABLE 6

Autoxidation of ascorbic acid

pH	DI-ANION %	MONO-ANION %	MOLECULE %	VELOCITY CONSTANT MIN.$^{-1}$ × 10^3	
9.21	0.5	99.5	—	22	No metallic ions
8.71	0.2	99.8	—	11	
7.61	0.01	99.99	—	0.7	
5.80	—	97.8	2.2	0.1	
4.70	—	79.4	20.6	0.03	
9.31	0.6	99.4	—	101	Copper-catalyzed
7.49	0.01	99.99	—	170	
6.12	—	99.0	1.0	134	
5.08	—	90.1	9.9	91	
3.87	—	36.0	64.0	26	
2.59	—	2.9	97.1	2	

* Weissberger & LuValle (63).

receptor combinations, because covalent bonds require a good deal of energy for breaking. In many cases, drug and receptor appear to be held together by ionic bonds reinforced by the so-called secondary linkages (hydrogen bonds and van der Waals' bonds). Hence changes in the pH of the medium bathing a receptor, whether inside the cell or outside, would be expected, in many cases, to bring about a change in biological response (see section 6). Conversely, a change in the degree, or even the kind, of biological effect can be expected from a drug employed at a new pH, or from an analogue of the drug, differing in pK_a.

Further, the examples given in Section 3 make it clear that each species of a substance (whether di-ion, mono-ion or molecule) has its individual chemical reactivity where the making and breaking of covalent bonds is concerned. Now the breaking of covalent bonds can bring about the degradation of a drug to a more biologically effective substance (*e.g.*, chloral hydrate to trichloroethyl alco-

hol pamaquine or its quinone, proguanil to its triazine) or to biologically inert material. Hence variations in pH or pK_a can influence the reactions between tissues and drugs and thus can control the nature and quantity of material to reach the receptors.

There are at least two other important ways in which ionization influences biological responses, *viz.*, adsorption at surfaces and the penetration of membranes.

(ii) *Adsorption at Surfaces*

Two main types of adsorption should be distinguished, (a) indiscriminate and (b) specific.

Indiscriminate adsorption, as shown by the common soaps, does not depend upon great affinity between the substance and the surface on which it becomes adsorbed. On the contrary, it depends mainly on the feeble ability of such substances (because of their predominantly hydrophobic nature) to unite with water molecules. If the hydrophobic "tails" are only a few carbon atoms long, they can be accommodated between the water-molecules, but molecules with a more markedly polar/non-polar character tend strongly to be squeezed out of solution by the high internal pressure of the water molecules which constantly tend to unite by hydrogen bonds with oxygen- or nitrogen-containing groups and, particularly, with other water molecules. Aqueous solutions of soaps, and of other indiscriminately adsorbable substances, are therefore squeezed out of aqueous solution, by the water molecules, on to any surface that presents itself whether it be the glass walls of the containing vessel, the air/water interface at the top of the solution or the skin of the experimenter's finger. Such deposits remain in dynamic equilibrium with the bulk and are usually only one or a few molecules thick. Ionization, usually, is unfavourable to such indiscriminate adsorption because the ion, being the more highly hydrated of the two species, has the greater tendency to remain in the water. Thus, from a dilute solution of soap, oleic acid is more strongly adsorbed than the oleate ion. The study of indiscriminate adsorption (*e.g.*, by means of surface-tension studies at the air/water interface) has gradually lost its fascination for students of drug-action in proportion as they have realized that indiscriminate adsorption is unlikely to lead to specific action.

Specific adsorption, on the other hand, is to be found among molecules that are quite well hydrated. It depends upon a complementariness of shape or charge between the drug and the receptor on which it exerts its disturbing effect. When the complementariness is one of charge, obviously the ion is more likely to be adsorbed than the molecule. The acridine antibacterials, discussed below, may be taken as examples of specific adsorption. More striking examples still are furnished by the vitamins of the B group which, perfectly water-soluble and occurring as they do in food at immense dilution, nevertheless become concentrated on their complementary surfaces, which are enzymes, with astonishing efficiency. This efficiency probably owes much to the universal phosphorylation of these substances which converts them into anions before they become co-enzymes.

(iii) *Penetration of Membranes*

Molecules with only one water-attracting group readily penetrate natural membranes provided that no more than 12 carbon-atoms are present (24). If, however, the water-attracting group is ionized, this penetration is retarded because the ionic group is held by unlike charges on the outer surface of the membrane and repelled by like charges. Davson and Danielli (24) quote many instances where the ionic form of a substance has been shown to penetrate membranes very much more slowly than the corresponding neutral molecule (see also 39). This phenomenon naturally brings to mind the ease with which neutrons, but not electrons or protons, penetrate the highly charged interior of atoms. It must not be supposed, however, that no ions ever penetrate natural membranes. In the first place specific mechanisms appear to exist for biologically useful ions (the human gut permits the ready passage of sodium and chloride ions but is relatively impervious to magnesium and sulphate ions). In the second place a non-penetrating ion can often be made penetrating by the addition of a lipophilic group; the chloro- and other lipophilic groups of the antimalarials mepacrine ("Atebrin") and chloroquine may assist thus in the necessary penetration of red blood cells.

A further interesting effect of ionization upon biological action can now be considered. The action of a drug obviously depends not only upon its ability to combine with some vitally important substance in a particular kind of tissue, but it also depends upon the ability of the drug to reach that tissue. Now a drug with a pK_a between 6 and 8 is in the interesting position that, at the physiologically important pH of 7, it is always in equilibrium with at least 10 per cent of its more poorly-represented ionic species. Such a drug will come to membranes through which only the non-ionic species can pass. Yet, as soon as this species has penetrated, it is likely to encounter an aqueous medium of similar pH value, and in this it is obliged to re-form ions until the same degree of ionization exists on both sides of the membrane. Quite a large number of drugs have pK_a values between 6 and 8, including the local anaesthetics and many common alkaloids (see Section 2 (v)). Some other families of drugs have pK_a values which lie entirely outside this range and hence they have a different pattern of distribution in the body, a pattern which may help to explain their different types of action.

(iv) *The Stability Constant (K_s) Governing Drug-Receptor Unions*

At one time it was thought that these questions of the degree of ionization would not have the importance in biology which they are now known to have. It was believed that in proportion as the biological receptors removed the toxic ionic species, more of it would be generated from the other species by the Mass Action Law, as in equation (ii) above. This may, of course, prove to be the case in some types of drug action not yet investigated. On the other hand, many examples are already known where biological activity is highly dependent upon the degree of ionization. This indicates that the combination between drug and receptor is usually weak and completely reversible in nature.

Let us consider, for example, a system where bacteria are in equilibrium with a low concentration of an antibacterial kation and a high concentration of the corresponding molecule which is non-antibacterial. In such a system, exhaustion of the kation by the bacteria would lead to the generation of significant supplies of kation from the molecule but the bacteria are themselves constantly shedding this kation back into the solution. Each bacterial anion (represented as $A^{\ominus}$), provided it is accessible to these kations, will enter into an equilibrium with them as in equation (vi) where K_s is the stability constant describing this equilibrium.

$$K_s = \frac{[ABH]}{[BH^{\oplus}][A^{\ominus}]} \qquad \text{(vi)}$$

ABH (the drug-receptor complex) is treated as an adsorption complex; it contains ionic bonds, but is also held together by secondary valencies as well. It may be visualized as a salt with a very high thermodynamic activity coefficient (see Section 2(i)).

When K_s and K_a are of similar magnitude, it is obvious that any deficiency of antibacterial kations ($BH^{\oplus}$) in the solution will be replenished from the drug-bacterium complex (ABH) as easily as from the non-ionized drug (B). Under these conditions the degree of ionization of the drug governs its efficiency; it is not enough that ions should be present, they must be present abundantly in order to force more $A^{\ominus}$ to become ABH.

The actual situation may be governed by several K_s values, because not all the kinds of groups (on the bacterium) with which the antibacterial combines are likely to be of vital importance for the existence of the bacterium. The combinations with the non-vital groups may be quantitatively great and can lead to the wastage of sub-optimal amounts of kation (50).

5. EXAMPLES OF SUBSTANCES WHOSE BIOLOGICAL ACTION DEPENDS UPON THE DEGREE OF IONIZATION

(i) *Substances which are Least Active when Ionized*

In 1921, Vermast noticed a tendency for weak acids to exhibit their biological activity most fully at those pHs where they were not ionized (62). One of the best authenticated cases of this kind is the inhibition of cell division in echinoderm eggs by salicylic acid (57). It can be seen from the upper curve of Fig. 2 that this acid is more active at pH 5 than at any other pH tested. At pH 5, a larger proportion of the salicylic acid ($pK_a = 3.0$) is in the form of molecules than at any higher pH, *viz.*, 0.99 per cent (see Table 4). When the concentration of salicylic acid present as molecules is calculated for each inhibitory concentration of total salicyclic acid (molecules + anions), the lower curve is obtained. It is evident from this lower curve that the inhibitory concentration of salicylic acid molecules remains the same regardless of pH; the only simple explanation is that the molecules, but not the anions, of salicylic acid inhibit the division of these eggs. It would be expected that at lower pH values salicylic acid would be a more powerful inhibitor because it would contain a higher ratio of molecules

co anions and one wonders why this region was not explored. However, complications may occur. Firstly the organism may not show the indifference to pH thange that it apparently does throughout the experimental range. Secondly the receptor for salicylic acid may undergo a change in ionization as the pH falls and would then hardly be likely to have the same affinity as before (see section 6).

Similarly, it has been found (19) that all members of a series of 30 barbiturates enter both eggs and larvae of the sea-urchin Arbacia *exclusively as molecules*. Moreover, the resulting depressions of cell division and of respiration were shown to be entirely due to molecules.

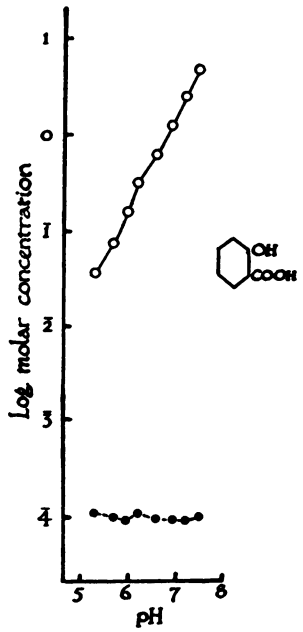


FIG. 2. The effect of pH on the concentrations of salicylic acid required to stop the cell division of *Echinarachnius parva*. Upper curve: total drug (= ions + molecules). Lower curve: molecules.

The action of *p*-aminobenzoic acid on the protozoon *Polytomella caeca* is of this kind. *p*-Aminobenzoic acid has a basic pK_a value of 2.65 (below this, it is largely kation) and an acidic pK_a value of 4.82 (above which it is largely anion). The maximal percentage of molecules occurs roughly at a pH equal to the average of these two figures, *viz.*, at pH 3.73. At this "isoelectric point", about 95 per cent of the acid is present in forms which are neither anions nor kations, *viz.*, about 85 per cent molecules and 10 per cent zwitterions (the latter are an ionic species to be discussed in Section 7 (ii)). It is interesting to see from Fig. 3 that the reversing action of *p*-aminobenzoic acid against sulphanilamide is greatest at this pH (43). For some other organisms (*E. coli*, *Aspergillus niger*), the anti-sulphanilamide action of *p*-aminobenzoic acid varies little with pH

which suggests that the anion and the molecule may have comparable effects in these cases (43).

(ii) *Substances which are Most Active when Ionized*

It is now known that many thousands of kinds of organic kations are antibacterial (1), but this knowledge does not extend back very far. In 1912, Morgan and Cooper (47) found that aliphatic amines (which exist mainly as kations at

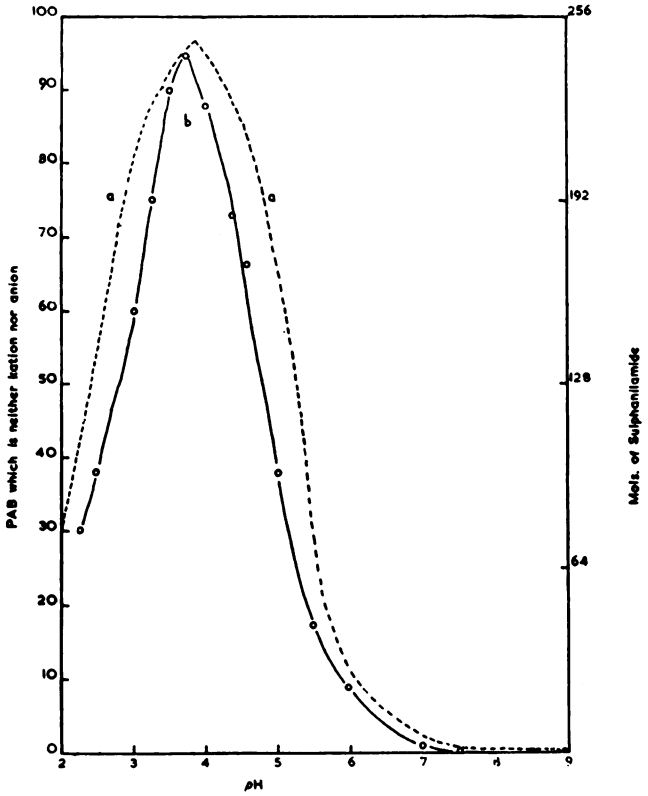


FIG. 3. Effect of *p*-aminobenzoic acid (PAB) in neutralizing the toxic action of sulphanilamide on *Polytomella caeca* at various pH values. (a) percentage of the PAB present as neither kation nor anion. (b) number of molecules of sulphanilamide antagonized by one molecule of PAB.

pH 7) were bactericidal and aromatic amines (which exist mainly as molecules at pH 7) were not. However, these authors did not sense the connexion between antisepsis and organic kations for they stated that antibacterial activity was due "to the presence of hydroxyl ions liberated through ionization of the alkylammonium hydroxides which are formed through the combination of the amines with water." In other words, they ascribed the effect to alkalinity. Had they used the same bases in the form of salts, or in a neutral buffer solution they would have found the same bactericidal properties. This was actually done by Tilley and

Schaffer in 1928 (60), but even then the obvious deduction was not made. However, in 1924 Stearn and Stearn (58) made their suggestion that organic kations (in particular those of the triphenyl-methane dyes) owe their antibacterial activity to a reaction with some anionic groups of bacteria to give feebly dissociated complexes of the type already discussed in Section 4 (iv) (see also 7 (iv)). Although the Stearns did not know the pK_a values of their dyes, they predicted that the salts of many strong (and fairly strong) bases would be found to be antibacterial because these would provide a sufficient supply of kations in the physiological pH range. They also showed that increasing the pH of the medium increases the antibacterial activity by bringing about increased (anionic) ionization of the acidic receptors of the bacterium. They pointed out that this alkalization of the medium must not be carried to the point where it begins to suppress the ionization of the antiseptic itself.

Rigorous proof of a connexion between kationic ionization and antibacterial action was first made in the acridine series (3, 7) which still provides the best examples of positive correlation between ionization and biological activity. Table 7 gives 26 examples of isomers in this series where the only difference between strong and weak antibacterial action is the difference between high and low degrees of (kationic) ionization. For example, five different mono-aminoacridines exist; of these, two are highly ionized at pH 7 because of an ionic resonance effect (5) whereas the other three ionize to only a small extent. It is evident from Table 7 that the two isomers which are well-ionized have a powerful antibacterial action whereas the three isomers that are poorly-ionized have only a feeble action. If we proceed to compare the well-ionized diaminoacridines with their poorly-ionized isomers, the same correlation is seen, and it is found again in the methyl-aminoacridines and in the chloro-aminoacridines, as Table 7 plainly shows. This correlation has been demonstrated in more than one hundred acridines and, provided ionization is kationic and is not allowed to fall below about 50 per cent at pH 7.3, the chemical nature of the substituents in the acridine nucleus make little difference (7).

Still more has been learned about the mode of action of acridines by the use of a test organism which withstands both acid and alkali (*E. coli*). This enabled a type of investigation to be made which has already been described for salicylic acid (Section 5 (i), above). This approach is complementary to that reported in Table 7. In such experiments, the ionization of the more poorly ionized acridines (*e.g.*, 3-aminoacridine) could be varied over the pH range of 5.5 to 8.3, and the effect on the organism observed. As a control, it was necessary to examine acridines which were fully ionized throughout this pH range (*e.g.*, 5-aminoacridine). The results of these experiments confirmed that the antibacterial action depended upon the kations rather than on the molecules (7). Further, the slope of the lines obtained when the logarithm of the antibacterial concentration of *kations* was plotted against pH (Fig. 4) revealed a direct competition between acridine kations and hydrogen ions. Naturally, no such correlation is seen when the logarithm of the antibacterial concentration of total drug (*i.e.*, kations + molecules) was plotted against pH (Fig. 5).

This correlation between kationic ionization and antibacterial activity exists

not only in the acridine series but also in at least seven other hetero-aromatic series. These seven series comprise the various angular and linear benzacridines, benzoquinolines and phenanthridines. Further, provided that appropriate substitution is used to keep the flat area of the molecule above 38 sq. A (in order to assist adsorption by van der Waals bonds), this correlation is valid also in the pyridine and quinoline series (6).

TABLE 7

Dependence of bacteriostasis on ionization in acridine derivatives

Minimum bacteriostatic concentration for *Streptococcus pyogenes* after 48 hours' incubation at 37°. Medium: 10 per cent serum broth. pH:7.3.

-ACRIDINE	CONCENTRATION	IONIZATION PER CENT
5-Amino-	1 in 160,000	100
2-Amino-	80,000	73
3-Amino-	10,000	2
4-Amino-	10,000	2
1-Amino-	5,000	<1
2:5-Diamino-	1 in 160,000	100
2:8-Diamino-	160,000	99
1:5-Diamino-	80,000	98
2:7-Diamino-	160,000	76
3:7-Diamino-	20,000	3
1:9-Diamino-	<5,000	<1
5-Amino-1-methyl-	1 in 320,000	100
5-Amino-4-methyl-	320,000	100
5-Amino-2-methyl-	160,000	100
5-Amino-3-methyl-	160,000	100
3-Amino-5-methyl-	20,000	3
4-Amino-1-methyl-	20,000	1
1-Amino-9-methyl-	<5,000	<1
5-Amino-2-chloro-	1 in 160,000	96
5-Amino-3-chloro-	160,000	94
5-Amino-4-chloro-	160,000	86
5-Amino-1-chloro-	80,000	83
2-Amino-8-chloro-	40,000	33
2-Amino-7-chloro-	40,000	20
2-Amino-5-chloro-	<5,000	11
3-Amino-8-chloro-	<5,000	<1

An interesting conclusion to be drawn from Fig. 4 is that a smaller amount of any aminoacridine would be effective in wounds if they were prevented from becoming acid. Clinical studies along these lines were made by the Australian Army during the last war and, as a result, it was found that better results were obtained when sodium bicarbonate lavage preceded treatment with aminacrine (5-aminoacridine hydrochloride).

That a high degree of ionization may be necessary for the activity of certain antimalarials was first suggested in 1937 by Christophers (14, 15). It was shown

by Gage in 1949 (26) that each of the four related substances ((III), (IV), (V) and (VI)) had two basic pK_a values. The first constant of those printed below each formula (and measured at 25°) relates to the aliphatic side chain;

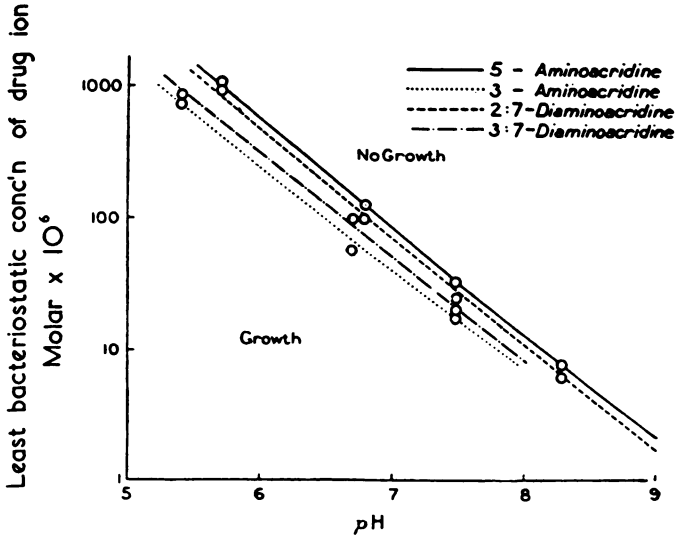


FIG. 4. Correlation in competition between hydrogen ions and acridine ions. Organism: *E. coli*.

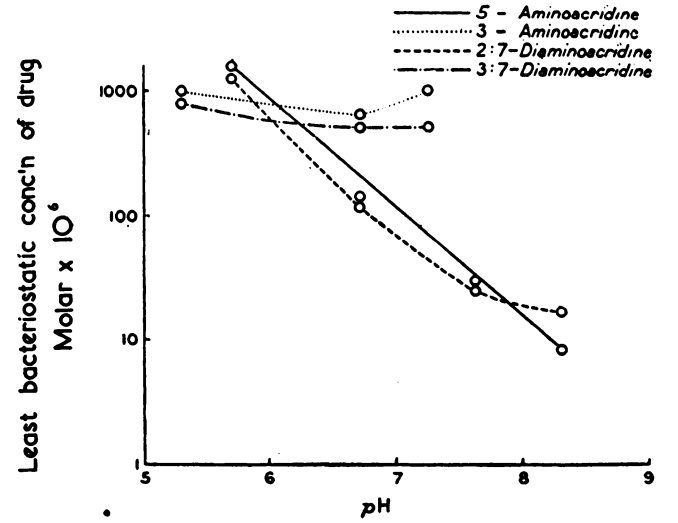
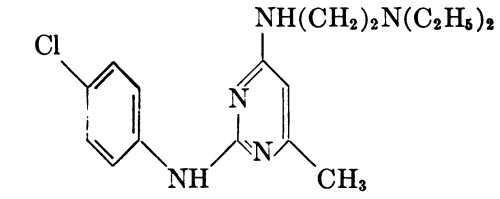


FIG. 5. Lack of correlation in competition between hydrogen ions and acridines (ions + molecules). Organism: *E. coli*.

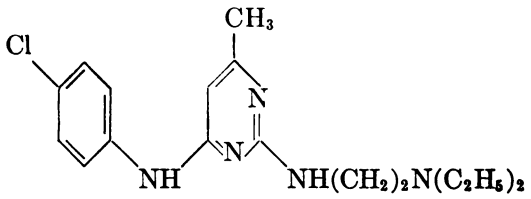
it is evident that this is completely ionized, in the blood, as a kation at pH 7.3. The second figure refers to the pyrimidine nucleus and the amino-group attached to it which collectively form an amidine system (53). Substances (III) and (IV) are highly antimalarial, substances (V) and (VI) are almost without antimalarial

action. Now at 37°, the second pK_a of substances (III) and (IV) becomes 6.4 (see Section 7 (i)) which, at pH 7.3, represents 11 per cent ionization as di-kation. Likewise substances (V) and (VI) would give 1 and 0.04 per cent of di-kations, respectively*. Rose based a general rule for predicting antimalarial activity in pyrimidines and diguanides (*e.g.*, proguanil) on these and similar results (53). However, he gave no clue as to whether analogues with higher second pK_a values were being prepared in order to obtain up to nine times as much of the desired ionic species.



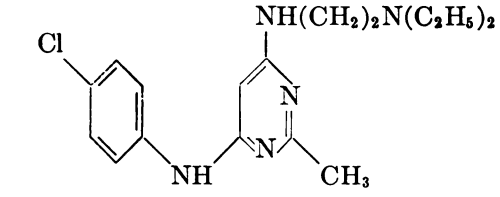
(pK_a: 9.05; 6.6)

(III)



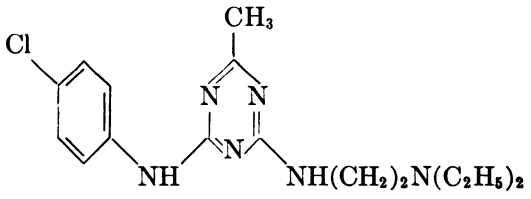
(9.6; 6.6)

(IV)



(9.3; 5.5)

(V)



(9.3; 4.05)

(VI)

* These calculations assume that the blood of the birds used in these experiments has the same pH as human blood. If it is not 7.3, but 7.0, the percentage of di-kation in substances (III) and IV) becomes 20, but those of the other substances are almost unaffected.

(iii) *Intermediate Cases*

A number of cases of biological action are known in which the molecule is far more active than the ion, but in which the ion does show some activity. Such behaviour is usually shown by phenols or other weak acids (4).

When submitting a weak acid to biological test, it is usually found that a constant amount of the substance is required to produce a standard response (regardless of the pH of the medium) at *all* pH values 1 unit or more below the pK_a. Under such conditions the ionization of the acid is slight (see Section 2 (vi)) and hence the biological effect is due to the molecule. This effect is illustrated on the left-hand side of Fig. 6. However if the pH is allowed to rise above the pK_a, an ever-increasing amount of the substance will be required to give the same response. When this response is analysed, one of two results is obtained, (a) a constant amount of the molecule is still required (*cf.* Section 5 (i) and Fig. 2) or (b) an ever decreasing amount of the molecule is required because the anion exhibits, to a limited extent, the biological action of the molecule. Result (b), which is illustrated in Fig. 6 by the action of benzoic acid on the mould *Mucor* (21), is far more common than result (a) (4).

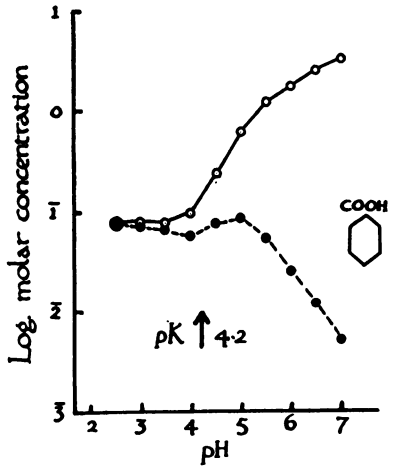


FIG. 6. The effect of pH on the concentrations of benzoic acid required to prevent the growth of *Mucor*. Upper curve: total substance (ions + molecules). Lower curve: molecules.

This method of plotting ionic data was developed by Simon (55) who found that the vast majority of those substances that are most active when least ionized nevertheless have ions which exert a small fraction of the activity of the molecules

Further examples of the increased efficacy of neutral molecules over their corresponding ions are provided in Table 8 which deals with the narcotizing action of various substances on the worm *Arenicola* (18). It can be seen that the effect of non-electrolytes such as chloroform is independent of pH. These non-ionizing substances provide valuable controls because they show that the changes of pH

do not affect the worms. Passing on to the weak bases, such as cocaine, we observe that these become more effective as the pH is increased, *i.e.*, in proportion as their ionization is suppressed. Similarly we note that the weak acids (for isomeric barbiturates) are more effective as the pH is decreased; again this corresponds to the suppression of their ionization. Actually (although the authors did not do so), it is a simple matter to calculate that in this series the ions are making a small contribution to the toxic action. Similarly, it has been shown that the inhibitory action of nitrophenols on fungi is due principally to the neutral molecules but that the ions do make some contribution (55). The chemotherapeutic action of phenylarsenoxides upon spirochaetes has also been shown to be due principally to the neutral molecules (25).

TABLE 8

The connexion between ionization and the narcosis of Arenicola

(Minimal anaesthetic doses, in grams per 100 ml. of sea-water, rendering this worm immobile after 5 minutes.)

	pH 7.0	pH 8.0	pH 9.0
Non-electrolytes			
*iso*Propyl alcohol	2.5	2.5	2.5
*iso*Amyl alcohol	0.1	0.1	0.1
Chloroform	0.012	0.012	0.025
Chlorbutol	0.025	0.025	0.025
Weak bases (pK_a = *ca.* 8.5)			
Cocaine	0.01	0.005	0.0025
Procaine	0.002	0.001	0.0005
Butyn	0.001	0.0002	0.0002
Barbituric acids (pK_a = *ca.* 8.0)			
*iso*Amyl, ethyl- ("amytal")	0.006	0.025	0.05
Propylmethylcarbinyl, ethyl ("nembutal")	0.003	0.006	0.012
Diethylcarbinyl, ethyl-	0.006	0.012	0.05
n-Amyl, ethyl-	0.006	0.012	0.05

This type of activity is not confined to weak acids. An investigation was made of the anaesthetic action (on the rabbit's cornea) of five local anaesthetics (cocaine, procaine, stovaine, β-eucaine and benzylbenzoylecgonine), all of which are weak bases (61). An analysis of the results along the lines of Fig. 6 shows that the biological activity is almost proportional to the amount of molecule present but that the kation does make some contribution.

Conversely, not all acids act as molecules. It is not possible for strong acids, which can exist only as anions in the physiological pH range, to form molecules. There is good evidence that the antibacterial alkylsulphonic acids and sulphates (*e.g.*, sodium cetyl sulphate) act by forming ionic bonds with some of the essential kations of bacteria (27, 46, 51). These antibacterials are the oppositely-charged analogues of the kationic antibacterials.

Sometimes the buffer solution, in which an experiment is performed, has a slight toxic action. As buffers are traditionally (but not of necessity) the salts

of weak acids, such toxic effects are commoner towards the lower pH values of each buffer's range. Sometimes this toxic effect is superimposed upon a curve which purports to show the effect of pH on the biological action of another substance (56). Such a source of error is revealed if buffers of various pK_a values are used. Thus the optimum for the growth of *Absidia orchidis* was found to be pH 4 when phosphate or citrate was used as a buffer; but with oxalate, which was slightly toxic, the optimum rose to pH 5 and with acetate, which was even more toxic, the optimum became pH 6 (22).

A curious situation arises among the sulphonamide drugs in that those members that are approximately 50 per cent ionized (as anions) at pH 7 are more powerfully antibacterial than such of their analogues as are ionized to a greater or lesser degree (8). One attempt at explanation assumed that the anionic form of the drug is the more potent but that the neutral molecule is essential to secure penetration (20). This explanation may well cover the phenols and other weak acids discussed above. However, in the case of the sulphonamides, the objection could well be made that these tests last for 24 hours or more, and hence good equilibration should be achieved; thus, much the same concentration of drug should be present both inside and outside the cell, almost regardless of the pK_a. Moreover, many closely-related substances which cannot dissociate into anions are valuable antibacterials, *e.g.*, sulphaguanidine and the various diphenylsulphones. A better explanation (8) has been put forward, *viz.*, that the really important thing is the size of the negative charge (on the SO_2-group) which appears to pass through a maximum value for those sulphonamides whose pK_a is approximately 7. This charge may assist adsorption through an ion-dipole bond on to the affected receptor, which appears to be the enzyme normally responsible for converting *p*-aminobenzoic acid to a member of the folic acid family.

6. THE IONIZATION OF RECEPTORS

A biological receptor, just as originally postulated by Ehrlich, is regarded as a molecule, or portion of a molecule, whose normal function in metabolism can be blocked by combination with an appropriate drug (*cf.* 4). There is evidence that receptors are often coenzymes or the protein components of enzyme systems.

The receptors which are relevant to the present review are those which can combine with drugs by ionic bonds, a type of receptor which only ionizable drugs can block. The pK_a values of various receptors cannot be predicted in advance of experiment for their chemical nature is largely unknown. Obviously, kationic drugs must combine with anionic receptors, but these may have pKs of 2 (due to the presence of phosphoric acid or amino-acid groups), 3 to 6 (carboxylic acid groups), 10 (tyrosine, purines, cysteine groups), etc. Kationic receptors could have pKs of 5 (pyridine groups), 7 (histidine), 10 (lysine), 13 (arginine), etc.

(i) *Receptors Outside Cells*

By no means are all receptors within cells. For example, amoebae are narcotized when undecane is injected into the external membrane, but not when it

is injected into the amoeba (44a). The frog's heart is affected (in opposite ways) by acetylcholine and methylene blue without penetration of the cells taking place (16). These and many similar observations are in harmony with current knowledge that the outside of a cell is rich in enzymes concerned with assimilatory and other processes. For example, yeast is known to have adenosinetriphosphatase and several hydrolases on the outer surface (54, 59).

The pK_a of a receptor on the outside of cells can often be studied by measuring the response to drugs over a range of pH values, provided that (a) the cell is known to be unaffected by the pH changes and (b) that the ionization of the drug does not change within this range (see Section 2 (vi)). For example, the effect of 5-aminoacridine on *E. coli* between pH 5.5 and 8.3 (see Fig. 4) shows that the drug is combining with a receptor which is ionizing more and more as the pH is raised. The drug has a pK_a of 10 and hence is completely ionized throughout this pH range. The rate of this increase in ionization (of the receptor) with increase in pH is undiminished at pH 8.3. As the increase would lessen when the pK_a of the receptor was reached, this pK_a must be 9 or more (3). That the receptors for kationic antibacterials are on the outside of cells is known from two facts: (a) examples with lipophilic groups are no more effective than those without such groups and (b) examples which yield 70 per cent of kation at pH 7 are no more active than those which yield practically 100 per cent (*cf.* Section 4 (iii)) (3).

Work on determining the pK_a of external receptors in this way is still in its infancy but has great potentialities for development from its early roots in the work of Stearn and Stearn in 1924 (*cf.* Section 5 (ii)).

Not all workers have given due consideration to the possibility that the receptor may change its degree of ionization as the pH is changed. For example eucupine (a derivative of quinine) was found to be more lethal to *Staph. aureus* at pH 8.6 than at 6.1 (45). After showing that the change in pH was not in itself injurious to the organism, the authors concluded that the active form of the drug must be the molecule and that it should be possible to make improved drugs in this series by the introduction of base-weakening groups into the molecule. They did not refer to the possibility that the effect of alkalinity on the action may be to increase the ionization of the relevant receptor; if this is so, the introduction of base-weakening groups would be undesirable. Again, in 1939 the relative toxicity of tyramine, benzylamine (and a number of related amines) for staphylococci was contrasted with that of their hydrochlorides in unbuffered media (see Section 2 (i)). Without measuring any pK_a values, the author concluded that the antibacterial action was due to the molecules rather than the kations. Clearly, the design of these experiments does not permit any such conclusion to be drawn.

Again, the respiration of avian red blood cells (whole or haemolysed) has been found to be inhibited 2.5 times as strongly by quinine at pH 10 as at pH 5. Because quinine has a pK_a of 8.4, it was concluded that the inhibition was caused by the molecule and not by the ion (by the base quinine and not by quinine salts, as the authors put it). The possibility that an acidic receptor ionizes at the higher pH (whereupon it can bind more quinine kations) is not mentioned (52).

(ii) *Receptors Inside Cells*

When any of the above tests suggests that a receptor is *within* the cell, ionization studies become more difficult. Obviously the pH surrounding the receptor is of prime importance, yet little is known of the pH within animal cells. Many experiments have been performed with indicators to determine the pH of protoplasm, but the results obtained are only a record of the average pH values of protein strands and other internal surfaces (see Section 7 (vi)). Some of these surfaces are likely to be poorer or richer in hydrogen ions than others, and they may not be at the pH of the internal bulk phase. This indicator method has suggested, rightly or wrongly, that the internal pH of animal cells tends to lie between 6.5 and 7. As the exact location of internal receptors is not known, this rough estimate may serve until more detailed knowledge is available.

That the pH within cells may roughly correspond to that of buffers in which they are placed is suggested by the following experiment. The tyrosine-decarboxylase of *Strept. faecalis* has itx maximal activity *in vitro* at pH 5.5. In the intact bacterium, it is only moderately active when the external environment is neutral or alkaline, but it becomes most active when placed in buffer at pH 5.0 to 5.5 (26a).

Thus it is desirable in pharmacology to work with a buffer, even when the receptor is known to be an internal one. Moreover the external effect of a buffer is desirable because it brings about uniform conditions by (a) presenting the drug to the cell in a standard state of ionization, regardless of the form in which it was supplied and (b) maintaining in a standard state the mechanisms for the adsorption of the drug to the cell surface and its penetration into the cell.

The success of buffering depends on the proportion of cells with which the buffers come in contact. In bulky tissue-preparations, this proportion may be very small, and the drugs can reach cells remote from the surface only after passage through other cells or intercellular fluids. The results from such experiments are not comparable with those obtained from experiments based on thin slices of tissue in which all the cells are in direct contact with the drug at a known pH (56).

7. FURTHER ASPECTS OF THE CHEMISTRY OF IONIZATION

The brief account of the chemistry of ionization given in Section 2 was intended to serve as a background for sections 3 to 6. However, there are other aspects of ionization chemistry which deserve mention because of the influence which they may exert upon the design of pharmacological experiments.

(i) *Temperature Effects*

The neutral point and many ionization constants vary with temperature. The neutral point is half the pK_w (defined in Section 2 (iv)). Thus neutrality occurs at pH 7.0 at 25°, but at pH 6.8 at 37°.

Bases become weaker as the temperature rises, and this effect is most marked with strong bases. Thus 0.011 is to be substracted for a base of pK_a 3 for each degree increase in temperature; for a base of pK_a 11 it becomes 0.022. Carboxylic

acids hardly vary in pK_a with temperature changes, but some other acids show changes similar to those of bases.

(ii) *Zwitterions*

So far, no mention has been made of zwitterions, *i.e.*, ionic forms which carry both positive and negative charges. In discussing whether a substance is zwitterionic or not, the pH range in which the information is required must be specified, for a sufficiently alkaline solution will change the zwitterion to an anion, and a sufficiently acid solution will change it to a kation.

For example, the acidic group in glycine has a pK_a of 2.2, and the basic group has a pK_a of 9.9. It is evident from Table 4 that at pH 1.2, only 10 per cent of the acidic groups (but *all* of the basic groups) will be ionized; hence at this pH, 10 per cent of the substance will be present as zwitterion and 90 per cent as kation. These calculations can be repeated at any other pH values which are of interest and it will be found that glycine is almost entirely zwitterionic between pH 3.3 and 8.9. Thus, a substance is 90 per cent or more in the zwitterionic state when the pH is at least 1 unit above the acidic pK_a and at least 1 unit below the basic pK_a. If this rule is applied to *p*-aminobenzoic acid (acidic pK_a 4.8; basic pK_a 2.7), it will at once be seen that this substance, unlike glycine, is exclusively anionic in neutral and alkaline solutions. At pH 3.8, solutions of *p*-aminobenzoic acid contain 90 per cent (neutral) molecules, 9 per cent zwitterions, and 1 per cent or less of kations or anions.

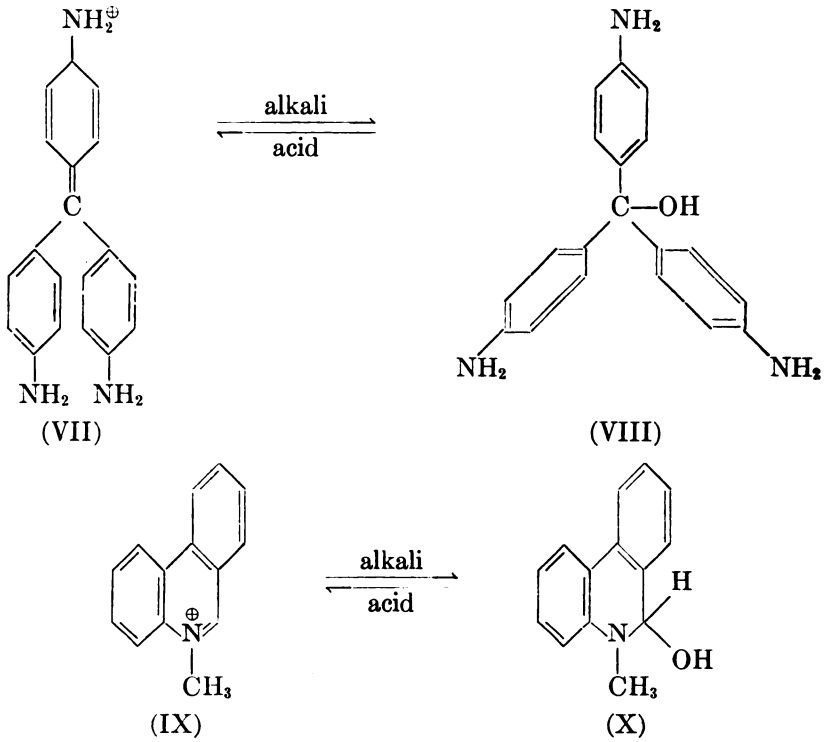
Zwitterions are usually rather inert pharmacologically. For example, histidine has none of the marked physiological properties shown by the closely related kation, histamine. The pharmacological properties of the histidine kation are unknown as this ionic species exists in quantity only below pH 2.

Again, the vinyl group of quinine can be oxidized to a carboxylic acid, thereby converting a kation to a zwitterion. Concomitantly, the antimalarial properties disappear. When this acid (quitenine) is esterified, the substance of necessity becomes kationic again; this ester is strongly antimalarial (30). Similar examples have been found in the acridine series (3).

(iii) *Pseudo-Bases*

The time taken for ionic equilibria to occur, in solution, is so exceedingly small that ionic reactions may be regarded as instantaneous. However, a limited variety of kations are capable of combining *covalently* with hydroxyl ions to give non-ionized substances known as "pseudo-bases" from which the original kation can be regenerated by acid. Although the reaction is governed by mass-action relationships, anything from an hour to a week may be needed for equilibrium to be achieved. The most common examples of pseudo-bases are furnished by heterocyclic quaternary compounds and by triphenylmethane dyes. Suitable methods for calculating the equilibrium ionization constants (expressed as pK_a^E) and the velocity constants at which equilibrium is obtained were recently devised and have been applied to the triphenylmethane dyes (29). These dyes undergo the transformation exemplified by parafuchsin, of which the kation (VII) is vul-

nerable to attack by hydroxyl ions, which gives the tertiary alcohol (VIII). Likewise heterocyclic quaternary compounds undergo the transformation exemplified by the kation of phenanthridine methochloride (IX), which gives the secondary alcohol (X). Several trypanocides belong to this class of substances.



(VII) (VIII)

(IX) (X)

It is not possible for the pharmacologist to correlate ionization with biological response if pseudo-bases enter the picture, unless he has first ascertained the pK_a at equilibrium and the time taken to achieve equilibrium. It may be advantageous to achieve equilibrium at a carefully chosen pH value before bringing the drug solution and the biological test-object together. It may be well to bear in mind that many secondary alcohols of the type (X) are easily oxidized to the corresponding ketones; each individual case can be tested for absorption of oxygen in a Warburg manometer. Few pK_a^E values of heterocyclic quaternary compounds have been obtained (29, 44) but it is evident that these are often much weaker bases than aliphatic and aromatic quaternary compounds; some aminotriphenylmethanes are very weak bases indeed. Unfortunately attempts have been made to explain the action of trypanocidal quaternary compounds without taking these factors into account (*e.g.*, 49).

A considerable amount of work has been done with triphenylmethane dyes in the field of therapeutic interference, and the experiments have involved estimating the amounts of various dyes taken up by cells or displacing one another from cell surfaces. Such colorimetric analyses have been carried out without any of the precautions outlined above and are of questionable validity. Doubts arise

even more strongly when alcohol has been used for extracting the dyes (36) as it rapidly etherifies alcohols of the types (VIII) and (X), thus furnishing a further barrier to equilibrium.

(iv) *Thermodynamic Activity Effects*

As was explained in Section 2 (i), anions and kations tend to form some ion-pairs in concentrated solutions and thus behave as though a (usually minute) fraction of their total concentration were absent. This thermodynamic activity effect (the activity being slightly less than the concentration) is reflected in pK_a values, both acids and bases appearing to be weaker in strong solutions. For example, acetic acid has the following pK_a values: 5.10 (M); 4.86 (0.1M); 4.79 (0.01M); 4.76 (0.001M). The value at infinite dilution can be calculated to be 4.75, and this is known as the thermodynamic pK.

Ions of formula-weight over 200 have a greater tendency to form ion-pairs (or even more complex aggregates) than the simple ions which we have been considering; hence these heavy ions show considerably greater activity effects, *i.e.*, the proportion of free to bound ions becomes very low. This must often be the case when a biologically active ion has combined with an oppositely charged receptor, particularly if the latter is part of a protein or a nucleic acid. The better the fit between the non-ionized parts of the two ions, the higher the activity effect (6). Although such combinations are as completely ionized as sodium chloride (Section 2 (i)), they provide only small amounts of free ions, the equilibrium being governed by a mass-action equation (see Section 4 (iv)). The size of K_s, the constant governing such an equation, is a measure of the strength of the non-ionic bonds cooperating with the ionic bonds in such a union. Such non-ionic bonds may be either hydrogen bonds, van der Waals' forces, or both, but they are not likely to be covalent bonds.

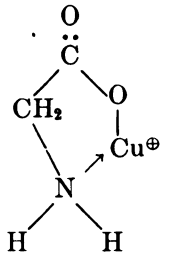
Drugs which are desired to provide a depot effect, *i.e.*, are required slowly to liberate a therapeutically-active ion, can be formed by combing such an ion with an oppositely charged ion, both being of fairly high molecular weight. Such compounds are slow to release the curative ions, because of their large thermodynamic activity effect. In addition, they are often (but not necessarily) sparingly soluble. Procaine penicillin is a well-known example.

(v) *The Binding of Metallic Ions*

Organic molecules having two neighbouring groups (one being an anion and one an electron donor such as a nitrogen or oxygen atom) form salts (with metallic ions) which show very high thermodynamic activity effects. That is to say, they are in equilibrium with very small amounts of the free metal ions. For example, the antiseptic 8-hydroxyquinoline combines with copper ions so that many millions of cupric kations are bound for each one that is free.

The name "chelation" was coined by Morgan and Drew (48) to describe this phenomenon, the name being derived from *chela* the crab's claw, in allusion to the shape and function of the metal-seizing pair of groups. A typical example of chelation is the union of one cupric ion with two anions of glycine to give the tightly-bound complex (XI). Complexes of this type play an enormous part in

biochemical processes. Their equilibria with the free ions from which they are derived can be described by mass action equations and simplified methods for calculating the required constants have recently been published (2).



(XI)

(vi) *The Zeta Potential* (ζ)

Those who are familiar with hydrogen-ion measuring equipment know that the $H^{\oplus}$ concentration is measured as a potential difference between two electrodes immersed in the solution. The greater the hydrogen ion concentration, the greater this potential. Conversely, if a potential exists between water and a surface immersed in the water, then the hydrogen ion concentration on the surface must be different from that in the water. Most biologically important surfaces contain polar atoms (O, N or S), and hence a small potential exists between the surfaces and water. This is the electrokinetic, or zeta, potential (ζ). Hartley and Roe (35) derived the following equation:

$$\text{pH (surface)} = \text{pH (solution)} + \frac{\zeta}{0.06} \qquad \text{(vii)}$$

(where the zeta potential is measured in volts). Zeta potentials of as much as 0.1 volt are common. Using a salt of cetanesulphonic acid to provide the necessary surface (in the form of colloidal particles dispersed in water), the authors showed that, while the pH of the solution was 8.0, that of the surface was 6.3. The difference between the two pH values decreased with rising concentration, the temperature being maintained at 30°. As biological surfaces are usually anionic, it must be expected that they will be at a lower pH than that of the buffers bathing them. Similarly, kationic surfaces should be at a higher pH than the buffers. What the magnitude of these differences is for proteins, nucleic acids and other biologically interesting colloidal material is almost unknown, but is not likely to exceed the values obtained in the above experiment.

The great majority of known dyes tend to congregate at surfaces, hence the limitations of indicators when used to determine the pH inside cells (*cf.* Section 6 (ii)).

(vii) *The Determination of pK_a Values*

From time to time the pharmacologist will need to know pK_a values which have not yet appeared in the literature. These values may be determined conductimetrically, spectrographically or potentiometrically. The last method is

least laborious. About 0.1 gram of substance is required; it must be completely pure and can be recovered unchanged after the titration (which is best carried out by a chemist who can apply all the necessary safeguards and corrections). For the potentiometric method to be applicable, the substance must be sufficiently soluble, both salt and molecule. It is difficult to find the pK_a of a sparingly soluble substance by titration if the value is low (it is usually not practicable to find a pK that is lower than the logarithm of the dilution employed). By the spectroscopic method, much more dilute solutions can be dealt with, but this method can be applied only to a substance whose ion and molecule have distinctly different spectra.

The following is a typical equation used in potentiometry (it is actually that for a weak base):

$$pK = pH - \log \frac{[B] + [H^+]}{[BH^+] - [H^+]} \qquad \text{(viii)}$$

where [B] and $[BH^+]$ are the apparent values calculated from the amount of acid added.

The pharmacologist who sends a specimen away for pK_a determination should ask to have the values reported to him at nine equidistant intervals (*viz.*, at 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent neutralization). Excluding the two extreme values (10 and 90 per cent), the other values should agree within ± 0.06. If this is not so, impurities are probably present, but occasionally the substance has decomposed during titration as is revealed by different values being obtainable upon back titration. Small credence should be given to pK_a values determined at "50 per cent neutralization" only, a method open to undetectable errors.

When two pK_a values, in the one substance, are separated by 3 units or less, the pH at 100 per cent neutralization of the first group should be numerically the average of the two pK_a values. When the pK_a values are 1.5 or less apart, equations of the type (vii) will not suffice, and the method of successive approximations must be applied. (For the method of successive approximations, see reference 9, p. 197.)

8. CONCLUDING REMARKS

Consideration of the principles discussed in this review makes it clear that it is highly advantageous for biologists to know the ionization constants of substances which they are investigating.

No less important is the next step which is to discover which is the most effective, the ion or the molecule. This may be done in two ways, which are not alternative but complementary. In the first approach, the pK_a of the drug should be varied by appropriate substitution, taking full advantage of the chemist's new-found ability to produce substances of any desired pK_a in almost any series. The advantage of this approach is that the ionization of the receptor is not interfered with, and the living cells are not removed from their optimal pH. The disadvantage is that the chemical change in the molecule may, of itself, be responsible for a changed biological response; this difficulty can be avoided by working with

two analogues at each desired pK_a, and accepting the results only if they agree. In the second approach, the composition of the drug should be kept constant, but the pH of the medium varied. This has the advantage that the living cells are exposed to only one drug, but the effect of pH changes on the receptor and on the viability of the cell itself must be independently examined.

Studies of this kind can add greatly to the scientific knowledge derivable from a biological experiment (and can assist in designing more meaningful experiments) because one of the commonest of variables is brought under control.

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