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# Multiple ionizations in proteins: Arsenical analogues of natural phosphates

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Abstract:
1. Handling multiple ionizations is facilitated by realizing that the titration of a substance with *n* ionizing groups is normally the sum of *n* one-site curves; the dissociation constants characterizing these curves are 'titration' constants. The pH dependence of any property is the sum of one-site curves with these constants. Each group in the molecule titrates in fractions, with a fraction titrating with each titration constant.
2. When enzymes that esterify, acylate or phosphorylate the -O-$PO_3H_2$ group of substrates act on analogues in which this group is replaced with -$CH_2$-$AsO_3H_2$, spontaneous hydrolysis follows the enzyme-catalysed reaction. This is because water easily replaces the alkoxy and acyloxy groups on arsenic that such enzymes introduce. Thus RNA polymerase and adenylate kinase effectively exhibit exonuclease and ATPase activities when given these analogues of diphosphate and AMP, respectively. A new consequence of enzymic transformations of such analogues is observed when glycerol-phosphate dehydrogenase oxidizes HO-$CH_2$-CH(OH)-$CH_2$-$CH_2$-$AsO_3H_2$, and when alcohol dehydrogenase oxidizes HO-$CH_2$-$CH_2$-$CH_2$-$AsO_3H_2$; the oxidation is followed by arsenite release, presumably a consequence of enolization of the ketone first formed.

## MULTIPLE IONIZATIONS IN PROTEINS

### Groups titrate in fractions

In teaching chemistry and biochemistry a lot of time is spent in teaching the properties of the rectangular hyperbola (Fig. 1a), the curve of the concentration of ligated form of a substance against the concentration of free ligand. This curve is often transformed by converting the horizontal axis to a logarithmic scale (Fig 1b). But just when we and our students have mastered such curves, we realize that they appear not to be applicable to the cases of most interest: to the groups in proteins whose behaviour depends on their ionic state. This is because as such a group titrates, its dissociation constant alters as each of the groups around it titrates. The purpose of the first part of this article is to show that the behaviour of such groups can indeed be described by curves like those of Fig. 1.

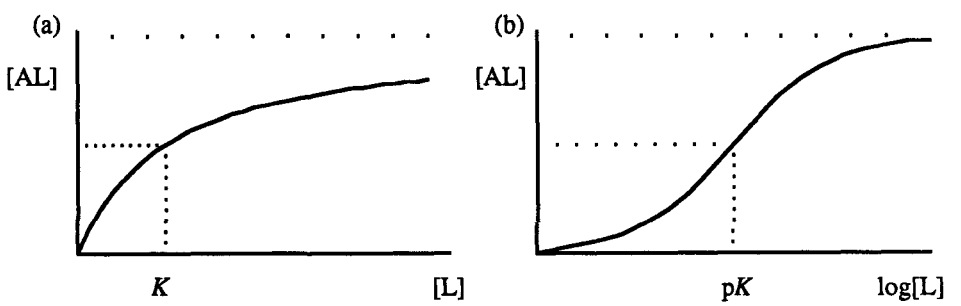



Fig. 1. Plots of the concentration of ligated form of a substance against (a) the concentration of free ligand, and (b) the logarithm of the concentration of free ligand.

As a first step in showing that the sigmoid shape of curve of Fig. 1b does help in portraying the behaviour of interacting groups, the example of a simple dibasic acid, $H_2Q$, such as malic acid, is used. Fig. 2a shows the dependence on pH of the concentrations of the species $H_2Q$, $HQ^-$, and $Q^{2-}$. I have chosen the molecular p$K$ values to be well separated, so that on raising the pH $[H_2Q]$ has become almost negligible before $[Q^{2-}]$ is appreciable. The curves intersect at pH = p$K_1$ and pH = p$K_2$, i.e when $[HQ^-] = [H_2Q]$ and when $[Q^{2-}] = [HQ^-]$, since $K_1 = [H^+][HQ^-]/[H_2Q]$ and $K_2 = [H^+][Q^{2-}]/[HQ^-]$.

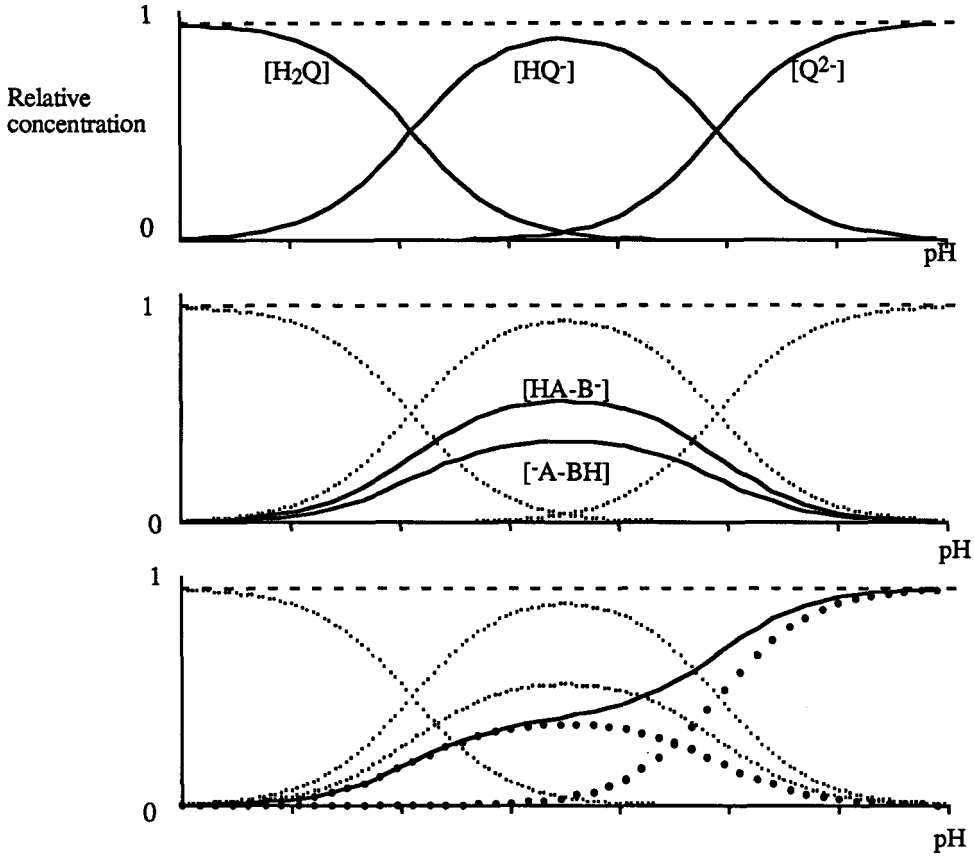



Fig. 2. pH Dependences of concentrations of forms of a dibasic acid. (a) The concentrations of $H_2Q$, $HQ^-$, and $Q^{2-}$. (b) The concentrations of the two forms of $HQ^-$, namely $HA\text{-}B^-$, and $^-A\text{-}BH$. The ratio between them is independent of pH. (c) The circles give the concentrations of $^-A\text{-}BH$ and $^-A\text{-}B^-$ from the previous graphs. Their sum, namely the fraction of the group -AH that is dissociated to $-A^-$, shown by the continuous line, is the sum of two one-site curves, such as Fig. 1b.

The species $HQ^-$, whose concentration is shown in the bell-shaped curve of Fig. 2a, can have two forms, according to whether it is one acid group, -AH, or the other, -BH, that is dissociated. Since the forms $HA\text{-}B^-$ and $^-A\text{-}BH$ have the same number of hydrons, the ratio between their concentrations is independent of pH. So their concentrations, which will be comparable if the acid strengths of the groups are comparable, are shown in Fig. 2b. The concentration of the ionized form of the group HA- is shown in Fig. 2c. From this it may be seen that the group -AH titrates in fractions, one with one p$K$ value, and one with another. Each fraction follows a curve of the type of Fig. 1b.

The fact (1,2) that each group in a substance with $n$ ionizing group titrates in $n$ fractions, each with a different p$K$, follows from the demonstration by Simms (3) in 1926 that the titration curve of the substance is identical to that of an equilmolar mixture of one-site acids; he called the constants for these hypothetical acids the 'titration' constants of the substance. This applies however the groups interact (so long as loss of one hydron does not facilitate loss of another). So the $n$ p$K$ values that the fractions of the groups possess are the same for all the groups; the groups differ in the fractions that have each of these constants.

This is an example of the fact that the pH dependence of <u>any</u> property of the substance is the sum of $n$ one-site curves of the form of Fig. 1b, with each curve characterized by one of the titration constants. The degree of dissociation of a group is just one such property.

The titration constants are found from the molecular dissociation constants $K_1$, $K_2$, $K_3$, etc. by solving the equation

$$x^n + K_1x^{n-1} + K_1K_2x^{n-2} + K_1K_2K_3x^{n-3} + \ldots = 0$$

The values of $x$ that are solutions are $-G_1$, $-G_2$, $-G_3$, etc., where each value of $G$ is a titration dissociation constant. In fact the titration p$K$ values (p$G$) differ negligibly from the molecular p$K$ values where each of these is a unit or more above the previous one.

The facts (a) that one hydron dissociates with each titration p$K$ (3), and (b) that one dissociates from each group, mean that a table can be constructed (1,2) with each column representing a titration p$K$ and each row a group; each cell contains the fraction of the group exhibiting that p$K$. The fact that rows and columns all add up to unity greatly lessens the number of assignments that have to be made to specify how each group in the molecule titrates. Thus for a molecule containing 10 groups, although the number of all group p$K$ values in the molecule is over 5 000 (i.e. $10 \times 2^9$), because each group has a different value according to the ionization state of each other group, and over 1 000 (i.e. $2^{10} - 1$) of these are separately assignable, a total of only 91 (i.e. $10^2 - 10 + 1$) assignments of p$K$ values and the fraction of each group possessing each of these values is enough to specify completely how each group titrates.

These results follow by mathematical identity from previous presentations. It could therefore be maintained that they are no advance, since nothing can follow from them that did not follow from the previous statements. Their advantage, however, is that they aid in thinking about the titration of groups. They emphasize that several groups may contribute to a single p$K$; hence it is often naive to ask which group in a molecule is responsible for a p$K$ that is observed in the change of some property with pH (e.g. enzyme activity), since several groups may contribute. Another example is shown on the debate on the p$K$ of about 6.5 shown in the $^{31}$P nmr spectrum of aspartate aminotransferase. The change in chemical shift seemed too small for the titration of $-PO_3H^-$ to $-PO_3^{2-}$. The debate is simplified (2) by pointing out that only part of the phospho group of pyridoxal phosphate may possess this p$K$, in agreement with earlier assignments (4); below this pH much of the group is already in the $-PO_3^{2-}$ form, and raising the pH through this p$K$ removes the hydron from the fraction still existing in the $-PO_3H^-$ form, as well as from a larger fraction of an adjacent group.

## ARSENICAL ANALOGUES OF NATURAL PHOSPHATES

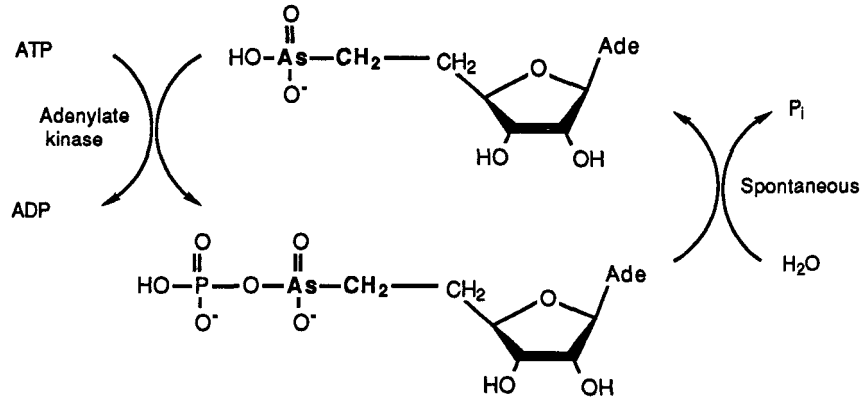
<u>Arsenates as analogues of phosphates</u>

Many examples are known of enzymes whose natural substrate is $R\text{-}O\text{-}PO_3H_2$ that act on the isosteric analogue $R\text{-}O\text{-}AsO_3H_2$. The study is not easy, because although $R\text{-}O\text{-}PO_3H_2$ has extremely stable P-O bonds, the arsenates do not. This is because the larger size of the arsenic atom allows water to enter its co-ordination shell and expel one of the four existing ligands. Most arsenates have half-lives in water at neutral pH of about 30 min (although this falls to seconds where R is acyl or $R'\text{-}PO_2^-$).

Our only contribution to this field was to show (5) that the apoenzyme of aspartate aminotransferase (EC 2.6.1.1), which can be reconverted into the active holoenzyme by the addition of pyridoxal phosphate, can similarly form an active and stable holoenzyme on treatment with pyridoxal and arsenate. Presumably the protein binds both pyridoxal and arsenate parts of the coenzyme so well that they spontaneously esterify, and that the tendency to hydrolyse is suppressed. The loss of activity was less than 10% in four days.

Arsonates whose enzymic transformation is followed by hydrolysis

If an enzyme, whose normal action is to alkylate, acylate or phosphorylate the phospho group of a substrate $R\text{-}O\text{-}PO_3H_2$, is given the substrate analogue $R\text{-}CH_2\text{-}AsO_3H_2$, and converts it into $R\text{-}CH_2\text{-}As(O)(OH)\text{-}OR'$, the spontaneous hydrolysis of this creates a futile cycle and so uses up the donor of the group put on. An example of this is the following reaction, in which the analogue of AMP effectively gives adenylate kinase (EC 2.7.4.3) ATPase activity (6):




Similarly, $H_2O_3As\text{-}CH_2\text{-}PO_3H_2$, by imitating diphosphate, can endow RNA polymerase (EC 2.7.7.6) with an artificial exonuclease activity (7), and $NH_2\text{-}CH_2\text{-}CH_2\text{-}AsO_3H_2$, by imitating both its natural substrates, $NH_2\text{-}CH_2\text{-}CH_2\text{-}O\text{-}PO_3H_2$ and the rarer one $NH_2\text{-}CH_2\text{-}CH_2\text{-}PO_3H_2$, causes ethanolamine-phosphate cytidylyltransferase (EC 2.7.7.14) to hydrolyse CTP to CMP and diphosphate (8).

These new futile cycles provide a new way of harming organisms, by using up the metabolites they synthesize at a high cost in energy, they might acquire chemotherapeutic uses if they could be selectively introduced into cells of tumours or pathogens.

Arsonates that eliminate arsenite

We have recently observed (9) that when glycerol-phosphate dehydrogenase acts on the arsonomethyl analogue of its substrate, namely $HO\text{-}CH_2\text{-}CH(OH)\text{-}CH_2\text{-}CH_2\text{-}AsO_3H_2$, arsenite is released, and the same happens when yeast alcohol dehydrogenase acts on $HO\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}AsO_3H_2$. It thus seems likely that the 3-oxoalkylarsonic acid formed by the enzyme enolizes and the enolate eliminates arsenite. This is a preliminary observation, but it raises the possibility that otherwise harmless arsonomethyl analogues of natural phosphates can be converted by enzymes into compounds that release arsenite, which is highly toxic by its binding to dihydrolipoyl groups of pyruvate and oxoglutarate dehydrogenases.

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