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Potentiometric Determination of the Second-Stage Dissociation Constants of Some Hydrogen Ion Buffers for Biological Research in Various Water + Organic Solvent Mixtures

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The second-stage dissociation constant values of 2-(*N*-morpholino)ethanesulfonic acid (MES), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) were determined at 25 ± 0.1 °C by potentiometric pH titration in pure water and various water + organic solvent mixtures. The organic solvents used are methanol, ethanol, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetone, and dioxane. The ionization of each of the zwitterionic buffers depends on both the proportion and the nature of the organic cosolvent used. It is observed that the pK_{a_2} values are slightly influenced as the solvent is enriched in methanol and ethanol and remain practically constant in the presence of different amounts of DMF and DMSO. A pronounced change in the pK_{a_2} values is observed as the solvent is enriched in acetone or dioxane. These results were discussed in terms of various solvent characteristics. It is concluded that the electrostatic effect has only a relatively small influence on the dissociation equilibrium of the imino group. Other solvent effects such as solvent basicity, different stabilization of the conjugate acid free base by hydrogen-bonding interactions in aquo + organic solvent media relative to pure aqueous media, and the proton–solvent interaction play an important role in the acid dissociation equilibrium.

Introduction

The choice of alternative buffers has increased with the commercial availability of zwitterionic amino acids, mainly N-substituted taurines or N-substituted glycines prepared by Good and co-workers (*1*) so that organic buffers suitable for use in biochemistry now include 2-(*N*-morpholino)ethanesulfonic acid (MES), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and *N*-[tris(hydroxymethyl)methyl]glycine (Tricine). Zwitterionic buffers—comparable to amino acids—show significant advantages over conventional buffers: insignificant penetration through a biological membrane, maximum buffer capacity at the physiological pH range 6.0–8.50, and no enzyme substrate or enzyme inhibitor properties. Though studies on the dissociation constants of acids and bases in mixed and nonaqueous solvents have been extensively investigated, relatively little work has been done to determine the dissociation constants of the biologically important zwitterionic buffers (*2–7*).

In the present paper the second-stage dissociation constants of MES, PIPES, ACES, HEPES, and Tricine have been determined in pure water and various water + organic solvent mixtures. The organic solvents used are methanol, ethanol, DMF, DMSO, acetone, and dioxane. The pK_{a_2} values have been discussed in terms of solvent properties.

Experimental Section

2-(*N*-Morpholino)ethanesulfonic acid (MES), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) were analytical grade (Merck) with a purity of 99% and were further purified according to Perrin (*8*). The organic solvents methanol, ethanol, DMF, DMSO, acetone, and dioxane were of high purity (A.R. or spectro grade products).

Carbonate-free NaOH was standardized by titration with potassium hydrogen phthalate. The molarity of HNO_3 was checked by titration with standard NaOH solution. Generally dilute solutions were prepared by appropriate dilutions of the stock.

Procedure. pH potentiometric measurements were made on solutions in a double-walled glass vessel at 25.0 ± 0.1 °C with a commercial Fisher combined electrode. The pH was measured with a Fisher Accumet pH/ion meter Model 2301 with a precision of 0.001 pH unit. The instrument was standardized against standard buffers of 4.0 and 9.20 pH values. The instrument was rechecked after each experiment. The temperature was controlled by circulation of water through the jacket, from a VEB Model E3E ultrathermostat bath, and maintained within ±0.1 °C. Purified nitrogen was bubbled through the solution in order to maintain an inert atmosphere. Efficient stirring of the solution was achieved with a magnetic stirrer. All test solutions were prepared in a constant ionic medium, 0.1 M KNO_3, by mixing the appropriate amounts of ligand, nitric acid, and potassium nitrate solutions. All the aqueous solutions of the different organic solvents were made by adjusting the total volume to 50 cm^3 by adding double-distilled water in each case. The concentration of hydrogen ion was decreased by the addition of sodium hydroxide, prepared in the ionic medium used for the solution. At each solvent percentage, at least 4 titrations (50 data points) were performed. The concentration of free hydrogen ion, *h*, at each point of the titration was calculated from the measured emf, *E*, of the cell RE/TS/GE (RE and GE denote the reference and glass electrode, respectively, and TS is the test solution) from the Nernst equation:

$$E = E° + Q \log h \qquad (1)$$

where $E°$ is a constant which includes the standard potential of the glass electrode and Q is the slope of the glass electrode response. It is to be assumed that the activity coefficient is constant, an assumption usually justified by performing the

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experiments with a medium of high ionic strength (0.1 M KNO_3) (*7, 9, 10*). The value of E_0 for the electrode was determined from a separate titration of nitric acid with potassium hydroxide, both of known concentration, under the same temperature and medium conditions as for the test solution titration. The data obtained were analyzed by the program MAGEC (*11*). During the MAGEC calculation the autoprotolysis constant of water, K_w, was refined until the best value for Q was obtained. This refined K_w value is necessary as an adjustable parameter in the pK_{a_2} calculation in pure water. Values for K_w for water in water + organic solvent systems have been taken from the literature (*12–15*). The pH meter readings have been corrected in accordance with the method described by Douheret (*16, 17*). Thus, if the pH meter is standardized using aqueous buffer, the meter reading pH(R) obtained in a partially aqueous medium differs by an amount δ from the corrected reading pH* which is referred to the standard state in the partially aqueous solvent, $pH^* = pH(R) - \delta$.

Initial estimates of pK_{a_2} values were refined with the ESAB2M computer program (*18*) by minimizing the error squares sum

$$U_v = \sum_i w_i (V_i - V_{calc,i})^2 \qquad (2)$$

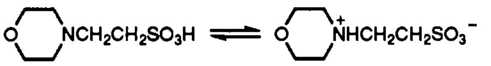
The weight is calculated by

$$1/W_i = S_i^2 = S_v^2 + (\delta V_i/\delta E_i)^2 S_E^2 \qquad (3)$$

The minimization function (2) has been chosen since the titrant volume, V_{calc}, can be calculated from an explicit equation (*19, 20*). The program ESAB2M minimizes (2) by using the Gauss–Newton nonlinear least-squares method (*21*) with the very efficient Levenberg–Marquardt algorithm (*22, 23*). In the potentiometric pH titrations the value $E° = 0$ has been used in the input instructions, since the program reads in pH in this case. The calculations have been performed with a Gaussian error in $V/S_v = 0.005$.

Results and Discussion

MES, PIPES, ACES, HEPES, and Tricine possess the following zwitterionic structures, respectively: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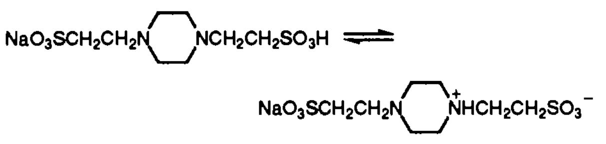
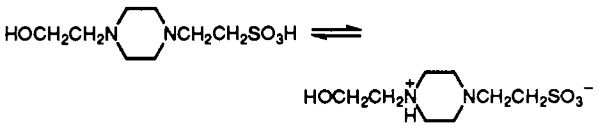

(5)


$$H_2NCOCH_2NHCH_2CH_2SO_3H \rightleftharpoons H_2NCOCH_2\overset{+}{N}H_2CH_2CH_2SO_3^- \qquad (6)$$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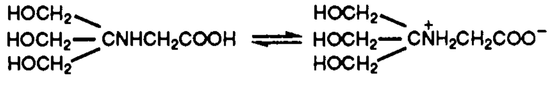

(8)

The second-stage dissociation constants of the above-mentioned zwitterionic buffers are due to cationic groups $-NH^+$ or $-NH_2^+$. The refined pK_{a_2} values of MES, PIPES, ACES, HEPES, and Tricine in the different aquo + organic solvent mixtures are given in Tables I–V. The values obtained in the present work for pK_{a_2} of the zwitterionic buffers studied in pure water agree with the literature data (*1*). With an increase in the amphiprotic methanol or ethanol solvent concentration in the aqueous medium, the change in $pK_{a_2}^*$ is quite small; in most cases it is slightly decreased. With an increase in the DMF or DMSO content, the $pK_{a_2}^*$ remains practically constant. By increasing the acetone or dioxane (nonpolar solvents) concentration in the aqueous medium, the $pK_{a_2}^*$ values increase.

Table I. Refined $pK_{a_2}^*$ Values of 2-(*N*-Morpholino)ethanesulfonic Acid (MES) in *w* Organic Solvent + (1 – *w*) Water Mixtures at 25.0 ± 0.1 °C

organic solvent	w	pK_{a_2}	$pK_{a_2}^{*\,a}$	S^a
methanol	0.00	6.115	6.115	0.002
	0.10	5.917	5.912	0.002
	0.20	5.980	5.965	0.003
	0.30	5.980	5.940	0.003
	0.40	5.905	5.815	0.004
	0.50	5.955	5.830	0.002
ethanol	0.00	6.115	6.115	0.002
	0.10	5.992	5.982	0.003
	0.20	5.967	5.932	0.004
	0.30	6.135	6.055	0.004
	0.40	6.167	6.035	0.003
	0.50	6.167	5.962	0.002
DMF	0.00	6.115	6.115	0.002
	0.10	6.110	6.048	0.003
	0.20	6.180	6.050	0.004
	0.30	6.217	5.992	0.002
	0.40	6.380	6.050	0.002
	0.50	6.530	6.078	0.003
DMSO	0.00	6.115	6.115	0.002
	0.10	6.117	6.085	0.003
	0.20	6.205	6.130	0.004
	0.30	6.292	6.132	0.002
	0.40	6.517	6.237	0.003
	0.50	6.580	6.155	0.004
	0.55	6.692	6.182	0.004
acetone	0.00	6.115	6.115	0.002
	0.10	6.167	6.187	0.004
	0.20	6.330	6.370	0.004
	0.30	6.355	6.405	0.003
	0.40	6.405	6.475	0.002
	0.50	6.521	6.642	0.002
dioxane	0.00	6.115	6.115	0.002
	0.10	6.142	6.162	0.003
	0.20	6.217	6.257	0.003
	0.30	6.392	6.472	0.003
	0.40	6.492	6.612	0.004
	0.50	6.805	6.975	0.005
	0.55	6.905	7.100	0.005

[a] $pK_{a_2}^*$: corrected pK_{a_2} values according to Douheret (*16, 17*). S refers to standard deviation.

Effect of the Amphiprotic Solvent. The proton activity a_H, as a measure of acidity, can be expressed (*24*) in terms of K_{HA} and K_{SH}, which are, respectively, the acidity constants of HA and SH_2^+:

$$a_H = [mK_{HA}K_{SH}(\nu_{SH_2^+}\nu_{HA}/\nu_{SH}\nu_{A^-})]^{1/2} \qquad (9)$$

where ν_{SH^+}, ν_{HA}, ν_{SH}, and ν_{A^-} are the activity coefficients of the species SH_2, HA, SH, and A, respectively, and m is the molality of HA.

A decrease in the dielectric constant usually causes $\nu_{SH_2^+}/\nu_{SH}$ and ν_{A^-}/ν_{HA} to increase, and it is possible that both of these ratios will increase by about the same amount (*25*). Hence, according to eq 9, a lowering of the dielectric constant due to addition of methanol or ethanol may have little effect on the acidity of an unbuffered solution of each of the zwitterionic buffers investigated. With respect to the solvent basicity effect, the autoprotolysis constant of water is slightly influenced by addition of methanol or ethanol (the pK of pure water is changed from 14.0 to 14.90 mol dm^{-3} for 0.5 mass fraction ethanol + water mixtures). Accordingly, one can deduce that changing the medium basicity by increasing

Table II. Refined pK_{a_2}* Values of Piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) in *w* Organic Solvent + (1 − *w*) Water Mixtures at 25.0 ± 0.1 °C

organic solvent	w	pK_{a_2}	$pK_{a_2}{}^{*a}$	S^a
methanol	0.00	6.768	6.768	0.002
	0.10	6.570	6.565	0.003
	0.20	6.633	6.618	0.004
	0.30	6.633	6.593	0.002
	0.40	6.556	6.466	0.002
	0.50	6.670	6.545	0.003
ethanol	0.00	6.768	6.768	0.002
	0.10	6.645	6.635	0.004
	0.20	6.620	6.585	0.002
	0.30	6.788	6.708	0.003
	0.40	6.820	6.688	0.003
	0.50	6.820	6.615	0.002
DMF	0.00	6.768	6.768	0.002
	0.10	6.763	6.701	0.003
	0.20	6.833	6.703	0.003
	0.30	6.870	6.645	0.004
	0.40	7.033	6.703	0.002
	0.50	7.183	6.731	0.004
DMSO	0.00	6.768	6.768	0.002
	0.10	6.772	6.740	0.003
	0.20	6.858	6.783	0.003
	0.30	6.945	6.785	0.002
	0.40	7.170	6.890	0.004
	0.50	7.233	6.808	0.003
	0.55	7.345	6.835	0.005
acetone	0.00	6.768	6.768	0.002
	0.10	6.820	6.840	0.002
	0.20	6.983	7.023	0.003
	0.30	7.008	7.058	0.004
	0.40	7.058	7.128	0.002
	0.50	7.174	7.295	0.003
dioxane	0.00	6.768	6.768	0.002
	0.10	6.795	6.815	0.003
	0.20	6.870	6.910	0.004
	0.30	7.045	7.125	0.004
	0.40	7.145	7.265	0.002
	0.50	7.458	7.628	0.003
	0.55	7.558	7.753	0.004

[a] pK_{a_2}*: corrected pK_{a_2} values according to Douheret (*16, 17*). *S* refers to standard deviation.

Table III. Refined pK_{a_2}* Values of *N*-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) in *w* Organic Solvent + (1 − *w*) Water Mixtures at 25.0 ± 0.1 °C

organic solvent	w	pK_{a_2}	$pK_{a_2}{}^{*a}$	S^a
methanol	0.00	6.830	6.830	0.002
	0.10	6.632	6.627	0.004
	0.20	6.695	6.680	0.004
	0.30	6.695	6.655	0.003
	0.40	6.620	6.530	0.002
	0.50	6.670	6.545	0.004
ethanol	0.00	6.830	6.830	0.002
	0.10	6.707	6.697	0.003
	0.20	6.682	6.647	0.004
	0.30	6.850	6.770	0.004
	0.40	6.882	6.750	0.003
	0.50	6.882	6.677	0.002
DMF	0.00	6.830	6.830	0.002
	0.10	6.825	6.763	0.003
	0.20	6.895	6.765	0.005
	0.30	6.932	6.707	0.004
	0.40	7.095	6.765	0.004
	0.50	7.245	6.793	0.003
DMSO	0.00	6.830	6.830	0.002
	0.10	6.832	6.800	0.003
	0.20	6.920	6.845	0.004
	0.30	7.007	6.847	0.005
	0.40	7.232	6.952	0.004
	0.50	7.295	6.870	0.004
	0.55	7.407	6.897	0.005
acetone	0.00	6.830	6.830	0.002
	0.10	6.882	6.902	0.003
	0.20	7.045	7.085	0.004
	0.30	7.070	7.120	0.005
	0.40	7.120	7.190	0.004
	0.50	7.236	7.357	0.005
dioxane	0.00	6.830	6.830	0.002
	0.10	6.857	6.877	0.002
	0.20	6.932	6.972	0.003
	0.30	7.107	7.187	0.004
	0.40	7.207	7.327	0.004
	0.50	7.520	7.690	0.002
	0.55	7.620	7.815	0.002

[a] pK_{a_2}*: corrected pK_{a_2} values according to Douheret (*16, 17*). *S* refers to standard deviation.

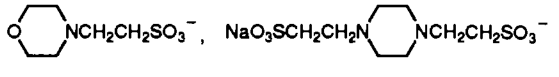
additions of methanol or ethanol to the aqueous medium has little influence on the second-stage dissociation constant of the zwitterionic buffers studied. Comparing the values of the pK_{a_2} of MES, PIPES, ACES, HEPES, and Tricine in 0.1 M KNO_3 water solution and at the same salt concentration, but in mixed water + ethanol or methanol solution, it can be seen that in ethanolic or methanolic solution the values of pK_{a_2} are only slightly changed compared with those for an aqueous medium. Such behavior is common for amino acids (*26*) and characterizes dipolar ions (*27*).

The results presented in Tables I–V, with respect to ethanolic solutions, can conveniently be discussed in terms of $\Delta G_{(\text{ion.})}$ defined as $2.303RT(\log K^{(w)} - \log K^{(s)})$, i.e., the difference between the standard free energies of ionization in the mixed solvent and in water (*27*). The ionization process can be represented by the general equation

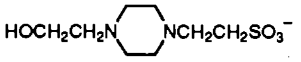
$$HA + SH \rightleftharpoons SH_2^+ + A^- \qquad (10)$$

where HS denotes a molecule of solvent. Since the solutes involved in the ionization are charged, then of the two basic steps in solute–solvent interactions, i.e., charge transfer and charge separation (*27*), only the first will be dominant. Therefore, although the difference in the dielectric constant between pure water and the water + ethanol mixture is appreciable (78.3 vs 49.0 at 25 °C), it will have little effect on the protonation constants of the solutes. Also, the solvation in mixed ethanol + water as solvent should not differ much from that in water, since the structures of the two solvents are similar. Consequently the two effects will result in relatively small values of $\Delta G_{(\text{ion.})}$.

Generally, it was recognized that properties of solvents such as the dielectric constant and acidic or basic strengths play a major but not exclusive role. Other factors such as stabilization of the different species existing in equilibrium through hydrogen bonding together with ion–solvent interaction and dispersion forces play an important role in the ionization of weak acids. Accordingly, the observed slight changes in pK_{a_2}* of MES, PIPES, ACES, HEPES, and Tricine as the solvent is enriched in methanol or ethanol can be mainly interpreted as resulting from the following two factors.



$H_2NCOCH_2NHCH_2CH_2SO_3^-$,



$$(HOCH_2)_3 \equiv CNHCH_2COO^-$$

(a) The relatively high stabilization of the conjugate bases by donor hydrogen bonds in a pure aqueous medium relative to that in the presence of methanol or ethanol. This is due to the greater tendency of water molecules to donate hydrogen as compared with other solvent molecules (*28*). Thus, an increase in the methanol or ethanol proportion in the aqueous

Table IV. Refined pK_{a_2}* Values of *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic Acid (HEPES) in *w* Organic Solvent + (1 − *w*) Water Mixtures at 25.0 ± 0.1 °C

organic solvent	w	pK_{a_2}	pK_{a_2}* [a]	S[a]
methanol	0.00	7.500	7.500	0.002
	0.10	7.453	7.448	0.004
	0.20	7.516	7.501	0.003
	0.30	7.514	7.474	0.005
	0.40	7.409	7.319	0.005
	0.50	7.459	7.334	0.002
ethanol	0.00	7.500	7.500	0.002
	0.10	7.377	7.367	0.003
	0.20	7.312	7.277	0.004
	0.30	7.480	7.400	0.004
	0.40	7.512	7.380	0.003
	0.50	7.512	7.307	0.003
DMF	0.00	7.500	7.500	0.002
	0.10	7.495	7.433	0.003
	0.20	7.565	7.435	0.003
	0.30	7.602	7.377	0.002
	0.40	7.765	7.435	0.002
	0.50	7.915	7.463	0.003
DMSO	0.00	7.500	7.500	0.002
	0.10	7.498	7.466	0.003
	0.20	7.586	7.511	0.004
	0.30	7.673	7.513	0.005
	0.40	7.989	7.618	0.002
	0.50	7.961	7.536	0.003
	0.55	8.073	7.653	0.002
acetone	0.00	7.500	7.500	0.002
	0.10	7.552	7.572	0.004
	0.20	7.715	7.755	0.002
	0.30	7.740	7.790	0.003
	0.40	7.790	7.860	0.002
	0.50	7.906	8.027	0.003
dioxane	0.00	7.500	7.500	0.002
	0.10	7.527	7.547	0.003
	0.20	7.602	7.642	0.003
	0.30	7.777	7.857	0.004
	0.40	7.877	7.997	0.004
	0.50	8.190	8.360	0.002
	0.55	8.290	8.485	0.003

[a] pK_{a_2}*: corrected pK_{a_2} values according to Douheret (*16*, *17*). S refers to standard deviation.

Table V. Refined pK_{a_2}* Values of *N*-[Tris(hydroxymethyl)methyl]glycine (Tricine) in *w* Organic Solvent + (1 − *w*) Water Mixtures at 25.0 ± 0.1 °C

organic solvent	w	pK_{a_2}	pK_{a_2}* [a]	S[a]
methanol	0.00	8.045	8.045	0.002
	0.10	7.795	7.790	0.003
	0.20	7.855	7.840	0.003
	0.30	7.855	7.815	0.004
	0.40	7.852	7.762	0.004
	0.50	7.875	7.750	0.003
ethanol	0.00	8.045	8.045	0.002
	0.10	7.825	7.815	0.003
	0.20	7.822	7.787	0.003
	0.30	7.965	7.885	0.002
	0.40	7.995	7.863	0.003
	0.50	7.995	7.790	0.004
DMF	0.00	8.045	8.045	0.002
	0.10	8.175	8.113	0.003
	0.20	8.245	8.115	0.003
	0.30	8.285	8.060	0.002
	0.40	8.445	8.115	0.003
	0.50	8.595	8.143	0.004
DMSO	0.00	8.045	8.045	0.002
	0.10	8.044	8.012	0.003
	0.20	8.133	8.058	0.003
	0.30	8.214	8.054	0.004
	0.40	8.443	8.163	0.004
	0.50	8.505	8.080	0.003
	0.55	8.615	8.105	0.003
acetone	0.00	8.045	8.045	0.002
	0.10	8.155	8.175	0.003
	0.20	8.365	8.405	0.003
	0.30	8.395	8.445	0.004
	0.40	8.455	8.525	0.003
	0.50	8.605	8.726	0.002
dioxane	0.00	8.045	8.045	0.002
	0.10	8.205	8.225	0.002
	0.20	8.215	8.255	0.003
	0.30	8.345	8.425	0.004
	0.40	8.395	8.515	0.003
	0.50	8.705	8.875	0.002
	0.55	8.835	8.030	0.005

[a] pK_{a_2}*: corrected pK_{a_2} values according to Douheret (*16*, *17*). S refers to standard deviation.

medium will result in an increase in the activity coefficient of the conjugate base, thereby causing a slight increase in the pK_{a_2} values.

(b) The greater stabilization of the proton in methanol or ethanol + water mixtures relative to that in pure water through ion–solvent interaction (*29*, *30*). This effect will generate a low activity coefficient of the proton in alcohol + water mixtures compared to that in a pure aqueous medium, therefore causing a slight decrease in pK_{a_2}.

Effect of Dipolar Aprotic Solvents (DMF and DMSO). The observed constancy in the pK_{a_2} values of MES, PIPES, ACES, HEPES, and Tricine in the presence of varying amounts of DMF and DMSO can mainly be explained as resulting from the following two opposing effects.

(a) A DMF + water or DMSO + water mixture is considered to be more basic than water (*31*). This behavior is based on the building up of a strong acceptor hydrogen bond from the $-NH^+$ or $-NH_2{}^+$ groups of the zwitterionic buffers studied in the former medium as compared to that in the latter one, thus facilitating the ionization process of $-NH^+$ or $-NH_2{}^+$ cationic groups, i.e., low pK_{a_2} values.

(b) There are some specific solute–solvent interactions among the conjugate free bases of each of 2-(*N*-morpholino)ethanesulfonic acid, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), *N*-(2-acetamido)-2-aminoethanesulfonic acid, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, and *N*-[tris(hydroxymethyl)methyl]glycine and solvent mixtures through hydrogen bonding. These are further strengthened for the conjugate free bases on their transfer from water to DMF or DMSO + water mixtures. This in turn results in low stabilization of these conjugate free bases and high pK_{a_2} values.

Effect of a Low Basic Aprotic Acetone Solvent. The presence of acetone as a coorganic solvent exerts a pronounced effect on the pK_{a_2} of MES, PIPES, ACES, HEPES, and Tricine as compared to the effect of the other coorganic solvents studied (methanol, ethanol, DMSO, and DMF). Though ethanol and acetone have nearly the same dielectric constants (24.30 and 20.70, respectively), the pK_{a_2} values of MES, PIPES, ACES, HEPES, and Tricine in ethanol and in acetone + water mixtures of the same composition are different. This behavior can be considered as convincing evidence for the above-reported conclusion that the electrostatic effect will have a relatively small influence on the second-stage dissociation constant of the zwitterionic buffers studied. Thus, the observed increase in pK_{a_2} when the amount of the coorganic solvent acetone in the medium is increased can be mainly attributed to the following effects.

(a) Solvent basicity effect: acetone is considered to be a very weakly basic solvent (*32*–*35*); therefore, one can expect that the basicity of acetone mixtures with water is lower than that of a pure aqueous medium (*31*). This leads to lower ionization of the NH^+ and $NH_2{}^+$ groups in the former media as compared to that in the latter one, i.e., a high pK_{a_2} value.

(b) Solute–solvent interaction effect: acetone is considered to be both a poorer acceptor as well as a poorer donor of hydrogen bonds compared to water (*36*). Thus, one may expect a low stabilization of the free conjugate bases of MES, PIPES, ACES, HEPES, and Tricine by hydrogen-bonding interactions in the presence of this coorganic solvent. This leads to higher pK_{a_2} values in such media than that obtained in pure aqueous solution.

(c) Proton–solvent interaction effect: it has been recognized that the hydrogen ion–solvent interaction plays a vital role in acid–base equilibria (*35*). Since acetone is a weakly basic solvent, the H^+ ion becomes less stabilized in the presence of this coorganic solvent. Thus, increasing the acetone concentration in the aqueous medium results in a high activity coefficient of the proton, i.e., a high pK_{a_2} value.

Effect of an Aprotic Nonionizing Dioxane Solvent. The observed inrease in the pK_{a_2} values of MES, PIPES, ACES, HEPES, and Tricine as the solvent is enriched in dioxane may be attributed to lowering of the dielectric constant which increases the fraction of associated ions to form Bjerrum ion pairs (*37*) and higher aggregates such as triple ions and dipole aggregates (*38*). In this aprotic nonionizing medium, the concentration of free ions is very low and acidity phenomena are governed largely by ionic association reactions, as Kolthoff and Bruckenstein have shown so convincingly (*39*, *40*).

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