Hydrogen Ion Buffers for Biological Research*

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ABSTRACT: Twelve new or little used hydrogen ion buffers covering the range $pK_a = 6.15–8.35$ have been prepared and tested. Ten are zwitterionic amino acids, either *N*-substituted taurines or *N*-substituted glycines, and two are cationic primary aliphatic amines. All of the zwitterionic buffers are better than conventional buffers in the Hill reaction and in the phosphorylation-coupled oxidation of succinate by bean mitochondria.

Two of the zwitterions, *N*-tris(hydroxymethyl)-methylaminoethanesulfonic acid and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, give particularly active and stable mitochondrial preparations. These two also give higher rates of protein synthesis in cell-free bacterial preparations than do tris-(hydroxymethyl)aminomethane (Tris) or phosphate buffers.

Too few substances suitable for use as hydrogen ion buffers between pH 6 and 8 have been accessible to biologists in the past. As a result some very inappropriate buffers have been used in biological research. These buffers have been bad because of inefficacy, undesired reactivity, or toxicity. Phosphate has poor buffering capacity above pH 7.5. It tends to precipitate most polyvalent cations and it is either a metabolite or an inhibitor in many systems. Tris-(hydroxymethyl)aminomethane (Tris) has poor buffering capacity below pH 7.5. Moreover it is a primary aliphatic amine of considerable reactivity and consequently it is often inhibitory. Borate is notorious for complexing a wide range of organic compounds including many respiratory intermediates. Glyclylglycine is an excellent buffer above pH 8 but it is almost useless below pH 7.5. It is also rather expensive. Other buffers such as imidazole, Veronal (5,5-diethylbarbiturate), maleate, and dimethylglutarate are for one reason or another even less satisfactory.

The inadequacy of our inventory of buffers may be a more serious problem than we realize. We cannot assume that various particulate systems and soluble enzyme systems all respond the same way to different pH-stabilizing addenda, and there may be no one ideal buffer for a given pH range. Unfortunately the number of available buffers is so limited that we have not been able to evaluate properly those we do have. It is impossible even to guess how many exploratory experiments have failed, how many reaction rates have been depressed, and how many processes have been distorted because of the imperfections of the buffers employed. This situation is unnecessary. Chemists have long understood, at least in a qualitative way, the relation of molecular structure to the chemical and physical properties of substances. It is easy to design and prepare a wide variety of inexpensive compounds with desired dissociation constants, solubilities, and reactivities. The following is a report on the synthesis, properties, and utility of some new buffers. Many more could be prepared, and we make no claims as to the universal superiority of the particular substances described.

In designing the new buffers the following rules of merit have been accepted. We realize, of course, that not all of these criteria are likely to be valid in all instances.

(1) The pK_a (*i.e.*, the pH of the midpoint of the buffering range) should be between 6 and 8 since this is the region of fewest buffers and most biological reactions.

(2) The buffer should have maximum water solubility and minimum solubility in all other solvents. Great water solubility is convenient in that it permits the use of concentrated buffer stocks. Much more important, however, is the ratio of the solubility in water to the solubility in relatively nonpolar solvents since this determines the distribution of buffer between the aqueous medium and the biological phase in particulate systems. Thus, for a given amount of buffering the concentration of buffer inside the cellular organelles is lower with more polar buffers.

(3) For the same reason (minimum extraneous material in the particles) the buffer should pass through biological membranes with difficulty.

(4) The buffer should produce a minimum of salt effects. If the system to be studied requires salts, suitable ions can be added, but if the system is adversely affected by salts, ionic buffers create problems.

(5) There should be a minimum influence of buffer concentration, temperature, and ionic composition of the medium on the dissociation of the buffer.

(6) Complexes formed with cations should be soluble

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and the binding constants of the complexes should be known. In the interest of simplicity, some of the buffers should not make such complexes.

(7) The buffers should be as stable as possible. They should resist enzymatic and nonenzymatic degradation under the conditions of use and they should not resemble enzyme substrates to the extent of acting as analog inhibitors.

(8) They should not absorb light in the visible or ultraviolet regions of the spectrum. Absorption at wavelengths greater than 230–240 mμ could interfere with widely used spectrophotometric assays.

(9) They should be easily prepared from inexpensive materials and should be easily purified by simple procedures such as recrystallization.

Some of these characteristics, extreme polarity, reduced membrane penetration, reduced ion effects, and crystallizability, are the characteristics of zwitterions. Other characteristics such as acid dissociation at and above neutrality, absence of ultraviolet absorption, and resistance to oxidation are characteristics of many secondary and tertiary aliphatic amines. Consequently most of the buffers described below are zwitterionic aliphatic amines, that is to say amino acids. The majority are either substituted glycines or substituted taurines.

It is not difficult to synthesize aliphatic amines with appropriate acid dissociation constants. Simple alkylamines are protonated far too readily for our purpose and therefore they only buffer at very low hydrogen ion concentrations. For instance, the pK_a of ethylamine is 10.75 and the pK_{a2} of glycine is 9.9. However, protonation becomes more difficult and the pK_a is lowered accordingly under a variety of circumstances. Thus the presence of the electronegative amido group adjacent to the nitrogen usually reduces the pK_a by 2.5–3 units. Several hydroxyls (as in Tris or triethanolamine) have a similar effect. Adjacent formal positive charges (as in half-protonated ethylenediamine) are responsible for an even larger lowering of the pK_a, about 3.5 units. The pK_a is also greatly lowered in those tertiary amines where the nitrogen is in a ring, since the protonated form is then considerably strained and therefore less easily formed. By taking advantage of these inductive, electrostatic, and steric influences, either singly or in combination, aliphatic amines can be prepared with pK_a values ranging from well below 3 to well above 11. Incorporation of at least one anionic group provides us with zwitterions capable of buffering in the desired pH range.

Twelve new or little used buffers are described below. Important physical and chemical properties, *i.e.*, pK_a values at different temperatures, metal–buffer binding constants, solubilities in water, etc., have been measured. We have also evaluated the new buffers by comparing them with standard buffers such as phosphate, Tris, and glycylglycine in three different biological systems. Our preliminary tests suggest that several of the new buffers may be superior to any of the buffers now widely employed.

Materials and Methods

Starting Materials. Details of the syntheses of the buffers will be presented in a separate section. The following is a list of all the starting materials with the commercial source of each: chloroacetamide, chloroacetic acid, and bromoacetic acid from Eastman Organic Chemicals, Rochester, N. Y.; morpholine, anhydrous trimethylamine, tris(hydroxymethyl)aminomethane, diethanolamine, and glycine from Fisher Scientific Co., Fairlawn, N. J.; anhydrous sodium sulfite and concentrated aqueous ammonia from J. T. Baker Chemical Co., Phillipsburg, N. J.; *N*-2-hydroxyethylpiperazine from Aldrich Chemical Co., Milwaukee, Wis.; piperazine hexahydrate from Nutritional Biochemicals Corp., Cleveland, Ohio; 1,2-dibromoethane from Matheson Coleman and Bell, Norwood, Ohio. Sodium 2-bromoethanesulfonate was prepared by the method of Marvel and Sparberg (1943).

Acid Dissociation Constants. Dissociation constants were determined by titrating the new buffers with a Radiometer automatic titrator-titrigraph which had been standardized by titrating well-known buffers under the same conditions. pK_a values were measured at several concentrations and several temperatures. Neutralization equivalents were determined at the same time with an error of about ±1%.

Metal Binding Constants. Approximate values for the metal–buffer binding constants were deduced from the displacement of the pH titration curve in the presence of an equivalent of the chloride salt of the metal in question. If we assume that the metal forms a coordinate bond with the amine nitrogen of the buffer and in so doing competes with protons, the following equations express the equilibrium condition

$$[\mathrm{A}] = [\mathrm{N}] + [\mathrm{NH}^+] + [\mathrm{NM}^+] \qquad (1)$$

where [A] is the total buffer concentration, [N] is the concentration of free amine, [NH$^+$] is the concentration of protonated amine, and [NM$^+$] is the concentration of metal–buffer complex. Obviously also

$$[\mathrm{M}] = [\mathrm{M}^{2+}] + [\mathrm{NM}^+] \qquad (2)$$

where [M] is the total metal concentration and [M^{2+}] is the concentration of free metal ion. Each of the two competing processes has its own equilibrium equation. Thus

$$K_a = [\mathrm{N}][\mathrm{H}^+]/[\mathrm{NH}^+] \qquad (3)$$

where K_a is the acid dissociation constant of the buffer and

$$K_M = [\mathrm{NM}^+]/[\mathrm{N}][\mathrm{M}^{2+}] \qquad (4)$$

where K_M is the metal–buffer binding constant. By substituting eq 1, 2, and 3 in eq 4, we obtain

$$K_M = \frac{[A] - K_a[NH^+]/[H^+] - [NH^+]}{(K_a[NH^+]/[H^+])([M] - [A] + K_a[NH^+]/[H^+] + [NH^+])} \quad (5)$$

Presumably all of the buffer is in the protonated form at the beginning of the titration, and therefore it is reasonable to assume that half is in the protonated form at the midpoint of the titration curve, or $[NH^+] = [A]/2$. Furthermore, in our experiments $[A] = [M]$. Thus for the midpoint of the titration curve eq 5 reduces to

$$K_M = \frac{2([H^+]/K_a - 1)}{[A](K_a/[H^+] + 1)} \quad (6)$$

The three values, $[H^+]$, K_a, and [A], are all provided by the titration curves observed in the presence and absence of the metal. Complex formation with magnesium, calcium, manganous, and cupric ions was investigated in this manner. When we report no metal binding we refer only to these four metals.

Other Properties. The melting points or decomposition points of the buffers were determined on a Fisher-Johns melting point block which had been calibrated with known substances. Solubilities of the buffers at 0° were determined by titrating aliquots of saturated solutions. Ultraviolet spectra of 0.05 M solutions of the buffers were determined with a Bausch and Lomb Spectronic 505 spectrophotometer.

Biological Tests. Reduction of potassium ferricyanide by illuminated chloroplasts (the Hill reaction) was measured spectrophotometrically by the method of Izawa and Good (1965). Oxidation of succinate by bean hypocotyl mitochondria (*Phaseolus vulgaris* L.) was measured as oxygen uptake in Warburg vessels at 20°. Protein synthesis in cell-free extracts of *Escherichia coli* was measured as incorporation of radioactive L-leucine. Details of the procedures are given in the Experimental Section.

Experimental Section

Preparation and Properties of the Buffers Tested. 2-(*N*-MORPHOLINO)ETHANESULFONIC ACID (Malkiel and Mason, 1943) was prepared by refluxing an aqueous solution of sodium bromoethanesulfonate with a large excess of morpholine for 2 hr. The greater part of the unreacted morpholine was removed by distillation at reduced pressure and the residue was taken up in water. Sodium ions and residual morpholine were removed by passing the resulting solution through a sulfonic acid resin column (Dowex 50) in the acid form. The eluate, which contained primarily product and hydrogen bromide, was taken almost to dryness by distillation at reduced pressure. The faintly colored product which precipitated on addition of alcohol was redissolved in hot alcohol containing a little water, decolorized with Norit, and recrystallized by cooling; yield 85%. A second recrystallization from alcohol and water gave a colorless material which decomposed above 300°; apparent pK_a when 0.1 M: 0°, 6.38; 20°, 6.15; 37°, 5.98; metal–buffer binding constant (log K_M) at 20° and 0.1 M: Mg^{2+}, 0.8; Ca^{2+}, 0.7; Mn^{2+}, 0.7; Cu^{2+}, negligible; effect of concentration on the apparent pK_a at 0°: 0.2 M, 6.17; 0.01 M, 6.18; concentration of a saturated aqueous solution at 0°, 0.65 M; proposed trivial name, MES.[1]

Anal. Calcd: N, 7.18; neut equiv, 195. Found: N (Kjeldahl), 7.06; neut equiv, 195 ± 2.

N-(2-ACETAMIDO)IMINODIACETIC ACID (Schwarzenbach *et al.*, 1955) was prepared by the reaction of 2 equiv of sodium bromoacetate with 1 equiv of glycinamide hydrochloride (see below) in water. A concentrated solution of NaOH was added at room temperature with stirring at a rate which maintained the pH between 7 and 8. After about 1.5 hr 3 equiv of NaOH had been added and the pH was steady at 8. The reaction mixture was then acidified to pH 2.2 with HCl, whereupon the very insoluble free acid precipitated and the pH rose. The mother liquors were again acidified to pH 2.0 and a second crop of the free acid precipitated. The product was purified by converting it into the very soluble monosodium salt and reprecipitating the free acid; yield (from chloroacetamide; see glycinamide below) 55%; decomp pt 220°; pK_{a1} below 2; pK_{a2} probably between 2 and 3; apparent pK_{a3} when 0.1 M: 0°, 6.85; 20°, 6.62; 37°, 6.45; metal–buffer binding constants (log K_M) as cited by Schwarzenbach *et al.* (1955): Mg^{2+}, 2.47; Ca^{2+}, 3.96; Mn^{2+}, 4.93; Cu^{2+}, 9.68; effect of concentration on apparent pK_a at 20°: 0.15 M, 6.60; 0.01 M, 6.78; solubility of free acid very small; solubility in buffering range (mono- and disodium salts) very high; proposed trivial name, ADA.

Anal. Calcd: neut equiv, 95. Found: neut equiv, 95 ± 1.

PIPERAZINE-*N*,*N*′-BIS(2-ETHANESULFONIC ACID) was prepared by refluxing a solution containing 2 equiv of sodium bromoethanesulfonate with 1 equiv of piperazine hexahydrate for 2 hr. After 0.5 hr a concentrated solution of NaOH was added dropwise at a rate which kept the pH of cooled, diluted aliquots between 9 and 10. When 2 equiv of alkali had been added and no more was being consumed, the reaction mixture was cooled and acidified to pH 1.5 with HCl. The very insoluble double zwitterion immediately precipitated; yield 60%. A small portion was recrystallized from boiling water (solubility about 1 g/l. at 100°). The greater part was purified by repeated conversions to the very soluble monosodium salt and reprecipitations of the acid.

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[1] Abbreviations used: MES, 2-(*N*-morpholino)ethanesulfonic acid; ADA, *N*-(2-acetamido)iminodiacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; Cholamine, (2-aminoethyl)trimethylammonium chloride hydrochloride; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Bicine, *N*,*N*-bis(2-hydroxyethyl)-glycine.

Another portion was converted to the monosodium salt and recrystallized from water and alcohol; decomp pt above 300°; $\mathrm{p}K_{a1}$ below 3; apparent $\mathrm{p}K_{a2}$ when 0.1 M: 0°, 7.02; 20°, 6.82; 37°, 6.70; no metal binding; effect of concentration on the apparent $\mathrm{p}K_{a2}$ at 20°: 0.2 M, 6.82; 0.01 M, 6.96; solubility in buffering range (mono- and disodium salts) very high; proposed trivial name, PIPES.

Anal. Calcd: N, 9.27; neut equiv, 151. Found: N (Kjeldahl), 9.20; neut equiv, 149.5 ± 1.5.

N-(2-ACETAMIDO)-2-AMINOETHANESULFONIC ACID was prepared by refluxing an aqueous solution containing 1 equiv of sodium bromoethanesulfonate with 1 equiv of glycinamide hydrochloride (see below). A concentrated solution of NaOH was added dropwise at a rate which kept the pH of cooled and diluted aliquots at about 8.3. After 2 equiv of NaOH had been added and no more alkali was consumed, the reaction mixture was cooled and acidified to pH 3 with HCl. On addition of one volume of alcohol the product precipitated; yield 50%. After recrystallization from hot water and alcohol, the product decomposed at 293°; apparent $\mathrm{p}K_a$ when 0.1 M: 0°, 7.32; 20°, 6.88; 37°, 6.56; metal–buffer binding constants (log K_M) at 20° and 0.1 M: Mg^{2+}, 0.4; Ca^{2+}, 0.4; Mn^{2+}, negligible; Cu^{2+}, 4.59; effect of concentration on the apparent $\mathrm{p}K_a$ at 20°: 0.2 M, 6.88; 0.01 M, 6.90; concentration of a saturated aqueous solution at 0°, 0.22 M; proposed trivial name, ACES.

Anal. Calcd: N, 15.38; neut equiv, 182. Found: N (Kjeldahl), 15.26; neut equiv, 182 ± 2.

(2-AMINOETHYL)TRIMETHYLAMMONIUM CHLORIDE HYDROCHLORIDE (Gabriel, 1920) was prepared by the action of excess concentrated aqueous ammonia on (2-bromoethyl)trimethylammonium bromide followed by an exchange of bromide ions for chloride ions on an amine resin in the chloride form. The (2-bromoethyl)-trimethylammonium bromide was prepared in good yield by the action of 1 equiv of anhydrous trimethylamine on 1 equiv of 1,2-dibromoethane in nitrobenzene. After recrystallization from alcohol, 1 equiv of the (2-bromoethyl)trimethylammonium salt was dissolved in 100 equiv of concentrated aqueous ammonia. After standing for 1 week at room temperature, the solution was taken to dryness by distillation at reduced pressure. The residue was recrystallized from alcohol, redissolved in water, and passed over a large amount of amine resin (Dowex 3) in the chloride form. The eluate was again taken to dryness and the residue was recrystallized from alcohol; decomp pt 260°; apparent $\mathrm{p}K_a$ when 0.1 M: 0°, 7.68; 20°, 7.10; 37°, 6.68; no metal binding; effect of concentration on apparent $\mathrm{p}K_a$ at 20°: 0.2 M, 7.18; 0.01 M, 6.88; concentration of a saturated aqueous solution at 0°, 4.2 M; trivial name from the literature, Cholamine.

Anal. Calcd: neut equiv, 175. Found: neut equiv, 175 ± 2.

N,N-BIS(2-HYDROXYETHYL)-2-AMINOETHANESULFONIC ACID (Izumi, 1954) was prepared by refluxing an aqueous solution of sodium bromoethanesulfonate with 2 equiv of diethanolamine for 2 hr. The cooled reaction mixture was passed over a sulfonic acid resin (Dowex 50) in the acid form. The eluate, containing product and HBr, was taken to dryness at reduced pressure and the product was recrystallized from aqueous alcohol; yield 52%; mp 153–155°; apparent $\mathrm{p}K_a$ when 0.1 M: 0°, 7.5; 20°, 7.17; 37°, 6.90; metal–buffer binding constants (log K_M) at 20° and 0.1 M: Mg^{2+}, Ca^{2+}, and Mn^{2+}, negligible; Cu^{2+}, 3.50; effect of concentration on the apparent $\mathrm{p}K_a$ at 20°: 0.2 M, 7.18; 0.01 M, 7.20; concentration of a saturated aqueous solution at 0°, 3.2 M; proposed trivial name, BES.

Anal. Calcd: N, 6.57; neut equiv, 213. Found: N (Kjeldahl), 6.54; neut equiv, 211 ± 2.

N-TRIS(HYDROXYMETHYL)METHYL-2-AMINOETHANESULFONIC ACID was prepared by refluxing an aqueous solution of sodium bromoethanesulfonate with 1.5 equiv of tris(hydroxymethyl)aminomethane (Tris) for 2 hr. The cooled reaction mixture was passed over a sulfonic acid resin (Dowex 50) in the acid form and the eluate was concentrated to a very small volume by distillation at reduced pressure. The product precipitated on addition of alcohol. It was redissolved in hot alcohol containing a little water and recrystallized by cooling; yield 60%; mp 226–228°; decomp pt 231°; apparent $\mathrm{p}K_a$ when 0.1 M: 0°, 7.92; 20°, 7.50; 37°, 7.14; metal–buffer binding constants (log K_M) at 20° and 0.1 M: Mg^{2+}, Ca^{2+}, and Mn^{2+}, negligible; Cu^{2+}, 3.80; effect of concentration on the apparent $\mathrm{p}K_a$ at 20°: 0.2 M, 7.50; 0.01 M, 7.54; concentration of a saturated aqueous solution at 0°, 2.6 M; proposed trivial name, TES.

Anal. Calcd: N, 6.11; neut equiv, 229. Found: N, (Kjeldahl), 6.09; neut equiv, 229 ± 2.

N-2-HYDROXYETHYLPIPERAZINE-*N'*-2-ETHANESULFONIC ACID was prepared by refluxing an aqueous solution of sodium bromoethanesulfonate with 1 equiv of *N*-2-hydroxyethylpiperazine for 2 hr. After 0.5 hr a concentrated solution of NaOH was added at a rate which kept the pH of cooled and diluted aliquots at about 9.0. When about 1 equiv of NaOH had been added and there was no further consumption of alkali, the reaction mixture was cooled and poured through a sulfonic acid resin (Dowex 50) column in the acid form. The column was washed thoroughly with distilled water and then eluted with an excess of dilute aqueous ammonia. Almost pure product came off the column as soon as the sulfonic acid of the resin was neutralized. The eluate was evaporated to dryness at reduced pressure, and the product was suspended in alcohol and a little water. Glacial acetic acid was added until aliquots of this suspension diluted with water were at pH 5.0, the isoelectric point of the product. The suspension was then cooled in an ice bath, filtered, redissolved in hot alcohol and water, and reprecipitated by cooling; yield 75%; mp 234°; $\mathrm{p}K_{a1}$ about 3; apparent $\mathrm{p}K_{a2}$ when 0.1 M: 0°, 7.85; 20°, 7.55; 37°, 7.31; no metal binding; effect of concentration on the apparent $\mathrm{p}K_{a2}$ at 20°: 0.2 M, 7.55; 0.01 M, 7.55; concentration of a saturated aqueous solution at 0°, 2.25 M; proposed trivial name, HEPES.

Anal. Calcd: N, 11.75; neut equiv (second acid dissociation only), 238. Found: N (Kjeldahl), 11.62; neut equiv, 238 ± 2.

N-(2-Acetamido)glycine was prepared by the action of sodium glycinate on chloroacetamide. Glycine (2 equiv) and 1 equiv of NaOH were dissolved in a very small volume of water at room temperature and 1 equiv of chloroacetamide was stirred in. A concentrated solution of NaOH was added at a rate which maintained the pH at 10. The temperature was not permitted to rise above 40°. After 2–3 hr, depending on the temperature, slightly more than 1 equiv of NaOH had been added and the chloroacetamide had all dissolved. Titration of the reaction mixture showed an 85% yield of a substance with $\mathrm{p}K_\mathrm{a}$ about 7.7. Acidification of the reaction mixture to pH 5.0 with acetic acid, removal of most of the water, and additions of successive amounts of alcohol yielded first the excess glycine and then the extremely soluble product badly contaminated with glycine. Recrystallization resulted in a preparation which was 80% product and 20% glycine. Since we have neither developed a practical synthesis of acetamidoglycine nor obtained a pure sample, we report here only its approximate $\mathrm{p}K_\mathrm{a2}$ and the fact that it shows some promise as a buffer for mitochondrial suspensions.

N-Tris(hydroxymethyl)methylglycine was prepared by the method previously described (Good, 1962); $\mathrm{p}K_\mathrm{a1}$ approximately 2.3; apparent $\mathrm{p}K_\mathrm{a2}$ when 0.1 M: 0°, 8.6; 20°, 8.15; 37°, 7.8; metal–buffer binding constants (log K_M) at 20° and 0.1 M: Mg^{2+}, 1.2; Ca^{2+}, 2.4; Mn^{2+}, 2.7; Cu^{2+}, 7.3; effect of concentration on the apparent $\mathrm{p}K_\mathrm{a2}$ at 20°: 0.2 M, 8.15; 0.01 M, 8.15; concentration of a saturated aqueous solution at 0°, 0.8 M; trivial name in the literature, Tricine.

Glycinamide hydrochloride was prepared by the action of a large excess of concentrated aqueous ammonia (100 equiv) on chloroacetamide. After 1 week at room temperature, the water was removed by distillation at reduced pressure and the product was recrystallized from alcohol. Apparent $\mathrm{p}K_\mathrm{a}$ when 0.1 M: 0°, 8.8; 20°, 8.2; 37°, 7.7; no metal binding; effect of concentration on the apparent $\mathrm{p}K_\mathrm{a}$ at 20°: 0.2 M, 8.25; 0.01 M, 8.15; concentration of a saturated aqueous solution at 0°, 4.6 M.

Tris(hydroxymethyl)aminomethane for biological research (Trizma Base) was obtained from Sigma Chemical Corp., St. Louis, Mo.; apparent $\mathrm{p}K_\mathrm{a}$ when 0.1 M: 0°, 8.9; 20°, 8.3; 37°, 7.9; negligible metal binding; effect of concentration on the apparent $\mathrm{p}K_\mathrm{a}$ at 20°: 0.2 M, 8.3; 0.01 M, 8.2; concentration of a saturated aqueous solution at 0°, 1.1 M; trivial name widely employed in the literature, Tris.

N,*N*-Bis(2-hydroxyethyl)glycine (Kiprianov, 1926) was prepared by the action of excess diethanolamine on bromoacetic acid in water. The product separated in good yield on the addition of alcohol to the reaction mixture; apparent $\mathrm{p}K_\mathrm{a2}$ when 0.1 M: 0°, 8.7; 20°, 8.35; 37°, 8.2; metal–buffer binding constants (log K_M) when 0.1 M: Mg^{2+}, 1.5; Ca^{2+}, 2.8; Mn^{2+}, 3.1; Cu^{2+}, 8.1; effect of concentration on the apparent $\mathrm{p}K_\mathrm{a2}$ at 20°: 0.2 M, 8.3; 0.01 M, 8.4; concentration of a saturated aqueous solution at 0°, 1.1 M; proposed trivial name, Bicine. The use of this compound as a buffer for biological investigation has been suggested by Remizov (1960).

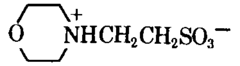
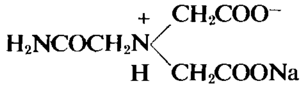
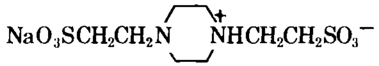
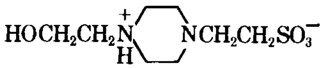
Glycylglycine was prepared by the action of a large excess of aqueous ammonia on chloroacetylglycine (Cheronis and Spitzmueller, 1941); apparent $\mathrm{p}K_\mathrm{a2}$ when 0.1 M: 0°, 9.0; 20°, 8.4; 37°, 7.9; metal–buffer binding constants (log K_M) at 20° and 0.1 M: Mg^{2+}, 0.8; Ca^{2+}, 0.8; Mn^{2+}, 1.7; Cu^{2+}, 5.8; effect of concentration on the apparent $\mathrm{p}K_\mathrm{a2}$ at 20°: 0.2 M, 8.4; 0.01 M, 8.4; concentration of a saturated aqueous solution at 0°, 1.1 M.

At buffering concentrations (0.05 M), our preparations of MES, PIPES, Cholamine, BES, TES, HEPES, Tricine, and Bicine absorbed a negligible amount of light at wavelengths greater than 240 mμ and very little light at 230 mμ. ACES absorbed significant amounts of light at 230 mμ (OD = 0.73) while our preparation of ADA absorbed light at wavelengths up to 260 mμ (OD = 0.21). To the best of our knowledge piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and *N*-(2-acetamido)glycine have not been described before.

We wish to emphasize that the data describing the physical properties of the buffers (summarized in Table I) have been selected on the basis of usefulness to biologists. The scope and accuracy of these data are not such as to commend our work to physical chemists, nor are our results couched in the thermodynamical terms they find convenient; rather we have presented the data with an eye to common biochemical procedures.

The Hill Reaction with Fifteen Different Buffers. Fresh leaves of spinach (*Spinacia oleracea* L.) were homogenized in a Waring Blendor for 10 sec at 0° in a medium containing 0.35 M NaCl, 0.05 M sodium phosphates, and 1 mM EDTA, pH 7.3. The homogenate was filtered through three layers of cheesecloth and centrifuged at 1500*g* for 5 min. The pellet was suspended in a medium containing 0.15 M sucrose and 0.05 M Tricine–NaOH, pH 7.4. After a brief centrifugation to remove cell debris, the chloroplasts were collected at 1500*g* (5 min) and resuspended in a small amount of the sucrose–Tricine buffer. Hill reaction rates were measured by following the reduction of potassium ferricyanide in a modified Bausch and Lomb Spectronic 505 spectrophotometer. Absorbance changes at 420 mμ were recorded on a strip-chart recorder. Actinic light of saturating intensity from a 500 w projector was passed through a red glass filter (transmission >600 mμ). The temperature was 15°. Reaction mixtures (2 ml) consisted of chloroplasts containing 30 μg of chlorophyll, 1.6 μmoles of potassium ferricyanide, the indicated amounts of the various buffers at pH 7.4, and small amounts of Tricine (5 μmoles) and sucrose (15 μmoles) introduced with the chloroplasts. For the measurement of electron transport uncoupled from

TABLE I: Physical Properties of Buffers.

No.	Structure	Proposed Name	pK_a at 20°	ΔpK_a/°C	Saturated Solution at 0° (M)	Metal–Buffer Binding Constants Log K_M			
						Mg^{2+}	Ca^{2+}	Mn^{2+}	Cu^{2+}
I		MES	6.15	−0.011	0.65	0.8	0.7	0.7	Negl
II		ADA	6.6	−0.011	—	2.5[a]	4.0[a]	4.9[a]	9.7[a]
III		PIPES	6.8	−0.0085	—	Negl	Negl	Negl	Negl
IV	$H_2NCOCH_2\overset{+}{N}H_2CH_2CH_2SO_3^-$	ACES	6.9	−0.020	0.22	0.4	0.4	Negl	4.6
V	$(CH_3)_3\overset{+}{\equiv}N—CH_2CH_2NH_2Cl^-$	Cholamine chloride	7.1	−0.027	4.2[b]	Negl	Negl	Negl	Negl
VI	$(HOCH_2CH_2)_2=\overset{+}{N}HCH_2CH_2SO_3^-$	BES	7.15	−0.016	3.2	Negl	Negl	Negl	3.5
VII	$(HOCH_2)_3\overset{+}{\equiv}NHCH_2CH_2SO_3^-$	TES	7.5	−0.020	2.6	Negl	Negl	Negl	3.2
VIII		HEPES	7.55	−0.014	2.25	Negl	Negl	Negl	Negl
IX	$H_2NCOCH_2\overset{+}{N}H_2CH_2COO^-$	Acetamido-glycine	7.7?	—	Very large	—	—	—	—
X	$(HOCH_2)_3\overset{+}{\equiv}CNH_2CH_2COO^-$	Tricine	8.15	−0.021	0.8	1.2	2.4	2.7	7.3
XI	$H_2NCOCH_2NH_2$	Glycinamide	8.2	−0.029	4.6[b]	—	—	—	—
XII	$(HOCH_2)_3\equiv CNH_2$	Tris	8.3	−0.031	2.4	Negl	Negl	Negl	—
XIII	$(HOCH_2CH_2)_2=\overset{+}{N}HCH_2COO^-$	Bicine	8.35	−0.018	1.1	1.5	2.8	3.1	8.1
XIV	$H_3\overset{+}{N}CH_2CONHCH_2COO^-$	Glycylglycine	8.4	−0.028	1.1	0.8	0.8	1.7	5.8

[a] Data of Schwarzenbach *et al.* (1955). [b] As the hydrochloride.

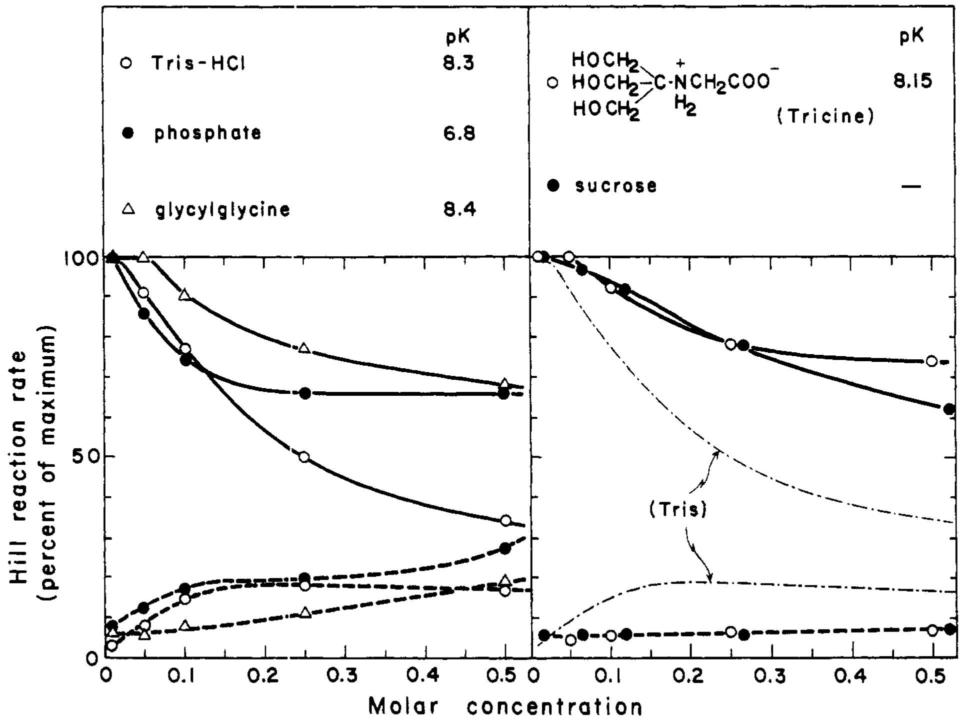


FIGURE 1: Rates of ferricyanide reduction by illuminated spinach chloroplasts with different buffers. Reaction mixture: chloroplasts containing 30 μg of chlorophyll, indicated amount of the various buffers at pH 7.4, 5 μmoles of Tricine and 15 μmoles of sucrose introduced from the chloroplast stock suspension, 1.6 μmoles of potassium ferricyanide, final volume 2 ml; light saturating; temperature 15°; solid lines, 100 μmoles of methylamine hydrochloride to uncouple electron transport from phosphorylation; broken lines, no methylamine. The maximum rate of ferricyanide reduction (100 on figures) was about 1000 μmoles/hr per mg of chlorophyll. All rates are compared to the rate with 0.05 M Tricine since no concentration of any other buffer gave higher rates. Note that all of the zwitterionic buffers uncouple and inhibit less than either the cationic or the anionic buffers.

phosphorylation, methylamine hydrochloride (100 μmoles) was added.

Figures 1, 2, and 3 are largely self-explanatory. The high rates (solid lines) are the methylamine-uncoupled rates and the low rates (broken lines) are the basal rates. A decrease in one of the high rates indicates inhibition and an increase in one of the low rates is presumably an expression of uncoupling. Clearly all of the buffers at concentrations above 0.1 M inhibit the uncoupled rate and many uncouple the basal rate. However, within these general similarities there are striking differences. The three zwitterionic-substituted glycines (glycylglycine, Tricine, and Bicine) inhibit to the same extent as sucrose and therefore we may assume that they inhibit solely by adversely affecting the osmolarity of the medium. These three have little or no uncoupling effect at any concentration. In contrast, the three cationic primary amines (Tris, Cholamine, and glycinamide) are disastrous at high concentrations; glycinamide is an uncoupler rivaling methylamine in potency, while at slightly higher concentrations it is an effective inhibitor. The substituted taurines (MES, ACES, BES, TES, and HEPES) are intermediate. Some are slightly more inhibitory in the uncoupled system than the substituted glycines or sucrose and all have slight uncoupling effects at high concentrations. It should be noted, however, that these taurines have lower pK_a values than the glycines and consequently a much higher proportion of the buffer is in the anionic form at pH 7.4. (The glycines with pK_a values above 8 are overwhelmingly in the zwitterionic form at pH 7.4.) The symmetrical disulfonic acid, PIPES, seems to be a rather effective uncoupler at high concentrations but this may reflect the presence of impurities in a not easily recrystallized substance. The anionic buffers, phosphate and maleate, uncouple slightly more than Tris, but are less inhibitory at high concentrations. (Bicine has the disadvantage for Hill reaction studies that it is slowly oxidized by ferricyanide at pH 7.4.)

We may sum up the comparison of the effects of the buffers on the Hill reaction as follows. All of the zwitterionic buffers are better than ionic buffers such as Tris, phosphate, etc., and the more of the buffer in the zwitterionic form the better it is. This applies both to the inhibition of already uncoupled electron transport and to the uncoupling of electron transport. The cationic primary amine buffers inhibit the uncoupled rate and uncouple the coupled system. The anionic buffers, as shown previously (Good, 1962), are uncouplers of photophosphorylation.

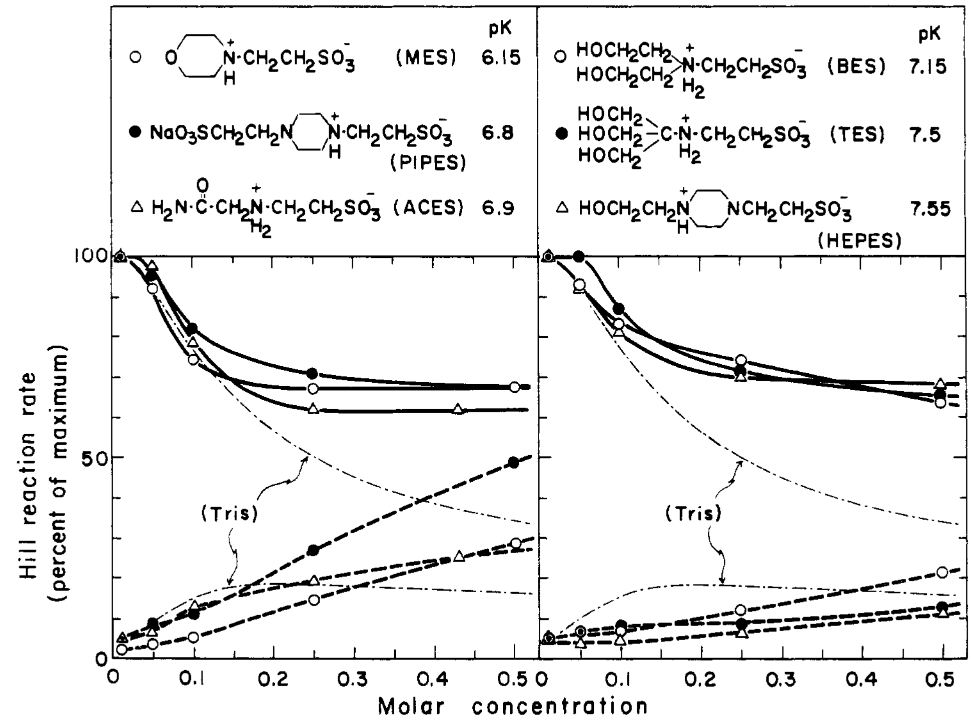


FIGURE 2: Rates of ferricyanide reduction. See caption to Figure 1 for conditions.

Phosphorylation-Coupled Oxidation of Succinate by Bean Mitochondria with Fifteen Different Buffers. Etiolated hypocotyls of pinto beans (*Phaseolus vulgaris* L.) were homogenized in a Waring Blendor for 15 sec (80 v) in the following medium: sucrose 0.25 M, EDTA 0.01 M, bovine serum albumin (fraction V powder, Pentex Inc., Kankakee, Ill.) 0.5%, and the indicated buffer 0.05 M, pH 7.4. Pellets were washed with the same medium lacking EDTA. The centrifugation procedure was a modified version of that of Lyons *et al.* (1964): low speed (2000*g* for 5 min), pellet discarded; high speed (17,300*g* for 10 min), supernatant discarded; another high speed, supernatant discarded; low speed (750*g* for 10 min), pellet discarded; another high speed, supernatant discarded. The final pellet was suspended in 2–3 ml of 0.25 M sucrose. Oxygen uptake was measured in Warburg manometers and phosphorylation was measured as disappearance of orthophosphate. The reaction mixture (3 ml) consisted of 450 μmoles of sucrose, 60 μmoles of phosphate, 15 μmoles of magnesium chloride, 6 μmoles of EDTA, 0.5 mg of hexokinase (Nutritional Biochemical Corp.), 60 μmoles of glucose, 0.1 mg of cytochrome *c*, 20 mg of bovine serum albumin (if used), 0.3 μmole of ADP, 0.3 mμmoles of ATP, 60 μmoles of sodium succinate, 60 μmoles (0.02 M) of the indicated buffer, pH 7.4, and mitochondria containing between 0.3 and 0.2 mg of protein. The temperature was 20°.

First, a preliminary screening of the buffers was undertaken. Each buffer was placed in a different vessel. Then the remaining components of the medium and mitochondria from a common stock were added. In this study the mitochondria had been prepared by grinding and centrifuging in Tricine buffer with serum albumin. Every buffer tested with the exception of maleate was better than Tris. This is not a measure of the excellence of the buffers but of the shortcomings of Tris since the reaction mixtures already contained phosphate, and phosphate alone gave rates 35% higher than Tris plus phosphate. The conclusion to be drawn from the first column of results in Table II is that most of these buffers are innocuous but superfluous in the already buffered medium. A few, such as ADA, ACES, and Cholamine, may be slightly inhibitory (much less so than Tris), whereas a few, such as TES, HEPES, acetamidoglycine, and glycylglycine, seem to be beneficial. The second column of Table II shows that serum albumin can protect the mitochondria, to some extent at least, from the harmful effects of Tris; the values in this column (which are relative to Tris) are higher than those in the first column, primarily because the rates with Tris were lower. However, the rates with HEPES and TES were, on an absolute basis, higher in the absence of serum albumin than in its presence. This raises again the interesting and as yet unsettled question of the nature of the mitochondrial deterioration which serum albumin arrests.

When we had established by these simple tests which of the new buffers showed promise, we began the much more laborious task of comparing rates of oxygen uptake and phosphorylation when the mitochondria had been isolated and stored in the selected buffers. In these experiments two new factors enter the picture: (a) the mitochondria are exposed from the first to the condi-

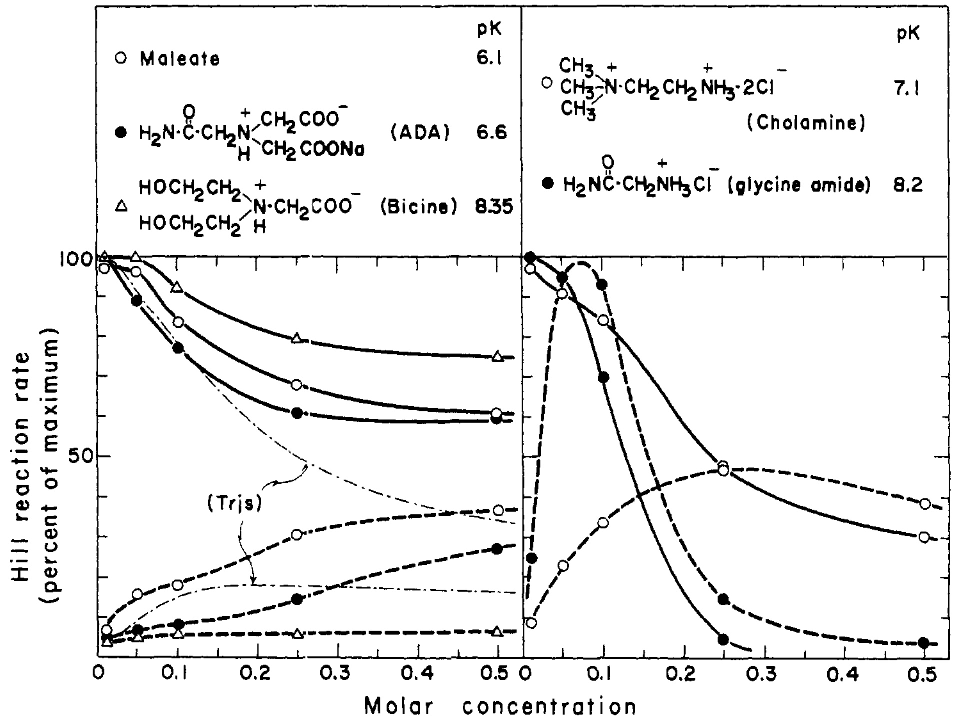


FIGURE 3: Rates of ferricyanide reduction. See caption to Figure 1 for conditions.

TABLE II: Succinate Oxidation by Bean Hypocotyl Mitochondria.

Buffer			Relative Rate of Oxygen Uptake (Tris = 100)		
			Mitochondria Prepared in Tricine + BSA		Mitochondria Prepared and Reaction Carried out in Test Buffer + BSA
No.	Name	pK_a at 20°	Reaction with BSA	Reaction without BSA	
I	MES	6.15	135	145	106
	Maleate	6.15	83	—	—
II	ADA	6.6	120	—	—
III	PIPES	6.8	130	—	112
	Phosphate	6.8	135	—	130
IV	ACES	6.9	120	125	112
V	Cholamine	7.1	120	—	150
VI	BES	7.15	130	145	130
VII	TES	7.5	140	165	270
VIII	HEPES	7.55	145	185	300
IX	Acetamidoglycine	7.7?	160	—	—
X	Tricine	8.15	125	150	125
XI	Glycinamide	8.2	105	—	—
XII	Bicine	8.35	150	—	93
XIII	Glycylglycine	8.4	160	210	—

tions we are interested in, and therefore we should expect to find the already observed differences exaggerated; (b) bean hypocotyls contain an appreciable amount of acid and thus the buffering capacity of the buffers at pH 7.4 is critical. Therefore, the values in the third column of Table II reflect both the buffering efficiency and the other merits of the buffer. MES fails miserably (being almost as bad as Tris) because, at pH

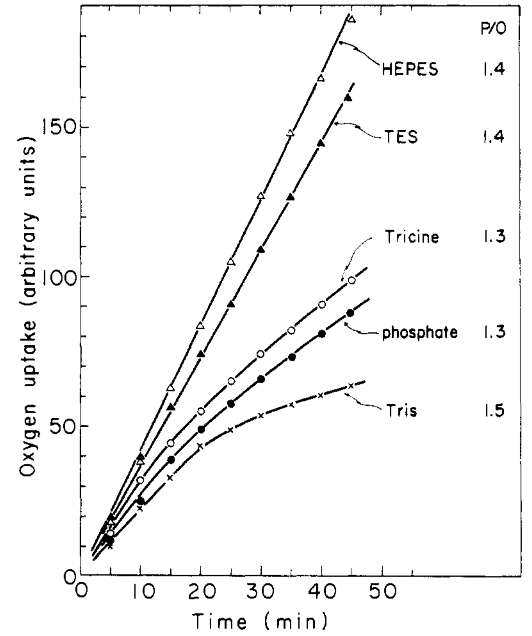


FIGURE 4: Phosphorylation-coupled oxidation of succinate by bean hypocotyl mitochondria with five different buffers. The tissues were homogenized in a medium consisting of 0.25 M sucrose, 0.01 M EDTA, 0.5% bovine serum albumin, and the indicated buffer, 0.05 M at pH 7.4. The mitochondria were isolated by differential centrifugation in the same medium without EDTA. The reaction mixture was as indicated in the text and lacked serum albumin; temperature 20°. The rate of oxygen uptake with HEPES was 750 μl of O_2/hr per mg of protein nitrogen.

7.4, it buffers so poorly, while the otherwise excellent Bicine is even worse than Tris because it completely fails to neutralize the acid released when the tissues are homogenized. TES and HEPES are outstanding, partly because they buffer so well (pK_a values = 7.5 and 7.55) and partly for other reasons we do not yet understand.

The P/O ratios averaged about 1.4 in these experiments. Phosphorylation efficiency did not vary consistently with rates of electron transport or with the different buffers. Moreover, none of the buffers caused a significant increase in the low rate of oxygen uptake in the absence of succinate. Consequently, the higher rates of electron transport encountered with some of the buffers cannot be attributed to uncoupling or to buffer oxidation.

Figure 4 shows the time course of succinate oxidation when the mitochondria were prepared and the reactions carried out in five different buffers. HEPES and TES seem to have positive protective actions beyond their buffering functions.

Incorporation of [^{14}C]L-*Leucine into Trichloroacetic Acid Precipitable Protein with Four Different Buffers. E. coli* cells growing exponentially in a salt–glucose medium supplemented with L-histidine, L-leucine, and uracil were harvested and washed in a buffer consisting of 0.01 M Tris–succinate at pH 7.4, 0.06 M potassium chloride, and 0.014 M magnesium acetate. The general procedure of Nirenberg (1963) was employed. Washed cells were passed through a French press at 16,000 psi and then centrifuged at 30,000*g* for 30 min to remove unbroken cells and cell debris. The supernatant fluid was dialyzed against 100 volumes of the same buffer for 8 hr. The resulting preparation is designated the "enzyme." The 1.0-ml reaction mixture used for measurement of [^{14}C]leucine incorporation contained the indicated buffer (0.1 M at pH 7.4) and, in μmoles, magnesium acetate, 60; potassium chloride, 50, manganous chloride, 2; ATP, 10; GTP, 5; CTP, 0.1; UTP, 0.1; phosphoenolpyruvate, 4; in μg, pyruvic kinase, 50; all the amino acids except leucine, 50. The content of radioactive leucine (specific activity 17.35 mcuries/mmole) was 38 mμmoles (205,000 cpm). The reaction tubes were preincubated for 3 min at 37° and then enzyme containing 3.8 mg of protein (Lowry *et al.*, 1951) was added, and the tubes were incubated for another 15 min at 37°. Control tubes were the same except that the preincubation and incubation were at 0°. The reaction was stopped by adding 5 ml of 5% TCA. Precipitated proteins were washed free from unincorporated amino acid as follows: the TCA-treated tubes were heated at 90° for 20 min, then cooled and centrifuged. The clear supernatant fluid was discarded and the precipitate was washed with another 5 ml of 5% TCA and then with 5 ml of a 3:1 mixture of alcohol and ether. The washed precipitate was resuspended in 5% TCA, decanted onto a Millipore filter, and washed another five times with 5-ml lots of 5% TCA. The filter containing the washed precipitate was dried in an empty scintillation vial for 15 min at 100°. Finally 15 ml of scintillation fluid [0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene in toluene] was put in the vials, and the radioactivity was determined in a liquid scintillation spectrometer.

The results with the different buffers are presented in Table III. Both TES and HEPES were significantly better than Tris–succinate. (In other experiments it was shown that Tris–succinate and Tris–HCl gave identical

TABLE III: Incorporation of [^{14}C]L-Leucine into Trichloroacetic Acid Precipitable Protein by a Cell-Free *E. coli* Preparation.

Buffer (0.1 M, pH 7.4)	L-Leucine Incorporated[a] (pmoles/mg of protein) Expt 1	Expt 2
Phosphate	36.1	38.6
Tris–succinate	46.0	45.0
TES	48.9	52.1
HEPES	53.9	51.9

[a] Values corrected by subtracting the small incorporation (0.22–0.31 pmole) which occurred at 0°.

results.) Phosphate was appreciably worse. It should be emphasized that in all of these instances the enzyme had a long exposure to Tris–succinate during its preparation and dialysis. Therefore it is not unreasonable to suppose that TES and HEPES might have given better protein synthesis rates if they had been used from the beginning of the preparation.

Discussion

There are two aspects to the evaluation of buffers—the question of their buffering capacities in the desired pH range and the entirely independent question of their side effects.

Evaluation of the buffering capacity of any buffer poses no problem if its pK_a, its concentration, and the operating pH are known; the adequacy of a buffer, as a buffer, depends only on the permissible concentration of the buffer and the nearness of the operating pH to the pK_a. Unfortunately, not all biologists are aware of the very significant effects of temperature on the pK_a values of many buffers. Thus, too often the worker who adjusts a Tris buffer to pH 7.8 at room temperature is unaware that he has prepared his material in the cold-room at pH 8.4 and assayed its activity in a 37° incubation at pH 7.4.

Evaluation of the side effects of buffers is a much more difficult task since these side effects may be different in every system studied. Moreover, differences in side effects can be obscured by differences in buffering efficiency. We must also consider that every buffer consists of two molecular species, protonated and nonprotonated, and these may have entirely different side effects. Fortunately it is possible to acquire considerable information about the nonbuffering effects of buffers by testing the substances in systems where there is little or no acid produced or consumed, or in systems where there is already adequate buffering, or in systems not very sensitive to pH change. Among our tests the Hill reaction (as we measure it) is rather indifferent to pH, and the mitochondrial system oxidizing succinate is already buffered with phosphate for phosphorylation. Since these two tests measure primarily side effects, the evaluations made in this paper often do not consider the pH-stabilizing capacity of the buffer. Thus when we say that HEPES is markedly superior to Tris we are not fully recognizing the additional superiority conferred by its better buffering action near neutrality. (Emphasis on testing the side effects of buffers has led us into some absurdities: it is ludicrous to compare Tris with MES as we have done, since their buffering ranges do not overlap and they could never be considered as alternatives. MES should have been compared with maleate, dimethylglutarate, histidine, and citrate.)

The criteria of suitability of buffers outlined in the introduction must be realistic since almost all of the buffers tested which conform to these criteria seem superior to the buffers which do not conform. Establishment of the general applicability of our criteria opens the way to the preparation of almost any number of other substances having good buffer characteristics. Syntheses like those described above are well within the ingenuity of biochemists, and we hope that an ever-widening assortment of buffers, tailored to specific applications, will be introduced by biochemists as needs arise.

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