Quantitative preparation of a TE/BSA buffer and of a graded sucrose series

Laboratory Exercise 1 | Integrated Course: Laboratory of Biochemical Methodologies and Experimental Models — Module of Biochemical Methodologies (BIOS-07/A, 5 ECTS) | Bachelor's Degree in Biotechnology, 2nd year, University of Teramo | Session date: 20 October 2026

1. Introduction to the technique

The subject of this exercise is the **quantitative preparation of solutions**: calculating how much solute a given concentration requires, weighing or pipetting it, dissolving it correctly and bringing the solution to a defined final volume. It is the operation that precedes almost every other laboratory procedure, and the one on which their results silently depend: a buffer prepared at the wrong concentration does not announce itself, it simply makes every subsequent measurement wrong by an amount nobody can reconstruct afterwards. For this reason the first laboratory session of the course was dedicated entirely to it.

Three relations cover the whole of the practical work:

1. Concentration expressed as **molarity** is the number of moles of solute per litre of solution, M = n/V = m/(MW · V), so the mass to weigh from a solid is m = M · MW · V.
2. Concentration expressed as **weight/volume percentage** is the number of grams of solute in 100 mL of final solution, % w/v = (g solute / mL solution) × 100, a unit preferred whenever the molar mass is irrelevant to the purpose — as it is for a protein additive such as albumin, which is heterogeneous, or for a bulk solute such as sucrose, whose role here is to set a density rather than to take part in a stoichiometry.
3. Preparation from a concentrated stock instead of from the solid conserves the number of moles, which gives the **dilution equation** and the dilution factor:

$$C_{conc}\,V_{conc} = C_{dil}\,V_{dil} \qquad DF = V_{final} / V_{withdrawn} = C_{initial} / C_{final}$$

A second theme runs through the exercise: a solution intended to come into contact with biological material cannot be prepared in distilled water. Water has no defined pH and no capacity to hold one — atmospheric CO_2 dissolving to carbonic acid is enough to bring it to pH 5.5–6.0 — and it offers no protection to the structures one is trying to preserve. A **buffer**, a solution containing a weak acid together with its conjugate base, resists the addition of acid or alkali because the added species is absorbed by the partner of the pair; the amount it can absorb before the pH moves appreciably is its **buffer capacity**, which increases with the concentration of the pair.

The solutions prepared here add two further protective functions to that basic one: a **chelating agent**, a molecule that binds a metal ion at several points at once and so withdraws it from solution, and a **sacrificial protein** that intercepts amphipathic contaminants and saturates adsorbing surfaces.

The specifications were not arbitrary. The solutions prepared in this session are the medium in which mitochondria will be isolated by centrifugation in a later session, and that intended use fixes every number in the protocol: a pH held near 7.4 at the working temperature of 4 °C, a medium free of divalent cations, an additive able to bind free fatty acids, and a series of sucrose concentrations whose densities span the buoyant density of the organelle, since a particle sediments only while its own density exceeds that of the surrounding medium and comes to rest where the two are equal. **The centrifugal separation itself, its theory and its results are the subject of a separate exercise and of a separate report**; what follows is confined to the preparation of the solutions and to the reasons the protocol specifies them as it does.

2. State of the art: how a fractionation medium is specified

Tris-HCl 10 mM. Tris (tris-hydroxymethyl-aminomethane) is the most widely used buffer in biochemistry between pH 7 and 9. A buffer is effective only within roughly one unit of its pK_a, which for Tris is 8.06 at 25 °C: the working pH of 7.4 therefore falls comfortably inside the useful window, whereas pH 6.0 would lie two units below it and the capacity would be negligible. The stock supplied is already titrated with hydrochloric acid, which is why it is designated Tris-HCl rather than Tris: both members of the conjugate pair are present in the bottle. A property of Tris that must be anticipated rather than discovered is the temperature dependence of its pK_a, which falls by about 0.03 units per degree of warming — so a solution titrated on the bench is substantially more alkaline once it is cooled, and the pH of a buffer destined for cold work has to be set at the temperature at which it will be used. Phosphate and the zwitterionic Good buffers (HEPES, PIPES) are the common alternatives and have much smaller temperature coefficients, but phosphate precipitates divalent cations and both interfere with some downstream assays; Tris remains the standard choice for subcellular fractionation.

EDTA 1 mM. Ethylenediaminetetraacetic acid carries six coordination sites — four carboxylate oxygens and two nitrogen atoms — which close around a metal ion like a claw, the image from which the word chelate derives. The multiplicity of bonds makes the complex far more stable than any single bond would be, and the stoichiometry is 1:1

irrespective of the charge of the ion. Its purpose in a fractionation medium is to remove the Ca^{2+} and Mg^{2+} liberated when tissue is disrupted, and with them the activity of the enzymes that require them: nucleases depend on Mg^{2+}, several proteases and the phospholipases on Ca^{2+}, and an excess of Ca^{2+} causes mitochondria to swell and lose function. The stock is supplied at pH 8.0 for two converging reasons: the free acid is practically insoluble in water and dissolves only when the pH is raised to about 8 with NaOH, and it is the deprotonated form that chelates effectively. The same chemistry explains EDTA blood collection tubes, in which sequestration of Ca^{2+} arrests the coagulation cascade. EGTA, which is far more selective for Ca^{2+} over Mg^{2+}, is preferred when magnesium-dependent activities must be retained.

BSA 0.1 % (w/v). Bovine serum albumin is abundant, inexpensive and without enzymatic activity, which makes it the standard sacrificial additive. It performs two distinct jobs. It binds the free fatty acids released from membrane lipids during homogenisation, which would otherwise insert into mitochondrial membranes, alter their permeability and uncouple respiration; and it coats the glass and plastic surfaces of tubes and tips, occupying the adsorption sites that would otherwise retain a significant proportion of a dilute sample. Working concentrations run from 0.1 to 0.5 % w/v according to the amount of tissue to be processed, and essentially fatty-acid-free grades are available where the scavenging function is the critical one. The corollary is that the medium itself contains 1 mg mL^{-1} of protein, a point returned to in Section 5.

Sucrose, 15 to 55 % (w/v). Sucrose is the reference density medium for organelles and membrane vesicles: non-ionic, chemically inert, non-penetrating, transparent, cheap and available at any concentration. An ideal gradient medium should cover the required density range while remaining soluble and forming a stable gradient, and should not become excessively viscous, shift the osmolarity, perturb the biology, prove toxic, interfere with downstream assays or attack the tubes. Sucrose satisfies most of these conditions but not all: at the concentrations needed to reach densities above 1.15 g cm^{-3} it is viscous and strongly hyperosmotic, which is why Percoll, Ficoll, Nycodenz and iodixanol — which deliver high density at low osmolarity — were developed, and why caesium chloride and sodium bromide or iodide are used for nucleic acids and lipoproteins instead. For a graded series prepared on the bench by weighing, sucrose remains the practical choice.

Preparing the solutions accurately. Volumetric glassware is calibrated by the manufacturer at 20 °C and marked class A, AS or B; class A and AS items are calibrated *to deliver*, so the volume dispensed is slightly less than the volume contained, the difference being the film of liquid left on the wall, while class B tolerances are twice as large. Balances are characterised by capacity and sensitivity, the smallest difference the instrument can resolve, and the uncertainty attributed to a reading is conventionally half that value: 0.005 g for a technical balance reading to 0.01 g, 0.05 mg for an analytical balance reading to 0.1 mg. The distinction between precision and accuracy belongs here as well — a sensitive balance that has not been levelled or calibrated returns closely agreeing readings that are all equally wrong — and it is the reason both balances are levelled on their adjustable feet and the analytical balance is used with its draught shield closed.

3. Materials and methods

3.1 Reagents, equipment and safety

Sucrose ($C_{12}H_{22}O_{11}$, MW 342.30 g mol^{-1}); Tris-HCl 1 M stock, pre-adjusted with hydrochloric acid; Na_2EDTA 160 mM stock at pH 8.0; bovine serum albumin powder; distilled water. Analytical balance (readability 0.1 mg) and technical balance (readability 10 mg), 100 mL graduated cylinder, beakers, weighing boats, spatula, adjustable micropipettes with disposable tips. A buttoned lab coat and nitrile gloves were worn throughout. The concentrated Tris and EDTA stocks are irritant to skin and eyes; sucrose is of low hazard but was handled with the same care; BSA is a very light powder and was weighed away from air currents and without inhaling it; the bench was kept clear, and every container was labelled with content, concentration, date and group before being filled.

3.2 Preparation of the TE buffer (100 mL)

The volumes of the two stocks were calculated with the dilution equation (Section 4.1). The 100 mL graduated cylinder was labelled and charged with approximately 60–70 mL of distilled water, that is, with less than the final volume, *before* any stock was added. This order matters for two reasons: the concentrated solutions are diluted immediately on entering rather than contacting dry glass, and the final adjustment becomes a genuine bring-to-volume rather than a sum of volumes, which would ignore the contraction that accompanies mixing. The calculated volume of Tris-HCl 1 M was pipetted into the cylinder; the tip was then discarded and replaced before the calculated volume of Na_2EDTA 160 mM was withdrawn and added to the same cylinder, since a tip carrying concentrated Tris would contaminate a stock bottle shared by the whole class. The two dilutions were thus performed simultaneously rather than separately, giving a single solution containing both components at their final concentrations — the TE buffer. The cylinder was swirled gently, brought to the 100 mL mark with distilled water with the bottom of the meniscus read at eye level, stoppered, inverted 10–15 times and stored at 4 °C.

3.3 Addition of BSA

The 100 mL of freshly prepared TE buffer was transferred to a clean beaker. The calculated mass of BSA was weighed on the analytical balance in a weighing boat, then sprinkled onto the surface of the buffer a little at a time and left to hydrate for a few minutes without stirring, so that the powder wetted on its own; the solution was then stirred very gently until clear. Neither vigorous shaking nor the vortex was used: albumin is surface-active, foams readily, and is denatured at the air–water interface. The container was labelled 'TE buffer + BSA 0.1 %' with date and group and stored at 4 °C, BSA being added on the day the buffer is to be used.

3.4 Preparation of the sucrose series (5 × 5 mL)

The five solutions were prepared in order of increasing concentration. For each, the balance was tared with the weighing boat, the calculated mass of sucrose was weighed and transferred to a beaker, and **TE + BSA buffer, not distilled water**, was added to no more than half the final volume — about 3 mL, and proportionally less for the more concentrated members, since the higher the concentration the less liquid can be added at the start without overshooting the volume. The mixture was stirred until completely dissolved, allowing a few extra seconds for the more concentrated solutions, which clear more slowly, and was then brought to the final 5 mL with the remaining buffer. Both the initial addition and the final bring-to-volume used the buffer, so that the solvent composition is identical in all five solutions and the only variable along the series is the sucrose concentration. The series consumes 25 mL of buffer in total, which was verified to be available before starting. All five were labelled and stored at 4 °C.

4. Results

4.1 The TE + BSA buffer

Applying $C_{conc}V_{conc} = C_{dil}V_{dil}$ to a final volume of 100 mL gives **1.00 mL** of the 1 M Tris-HCl stock, a 100-fold dilution, and **0.625 mL**, i.e. 625 µL, of the 160 mM Na_2EDTA stock, a 160-fold dilution; the remaining 98.4 mL is the distilled water added as the bring-to-volume. From m = (% w/v × V_{mL})/100, BSA at 0.1 % w/v in 100 mL corresponds to **0.100 g**, that is 100 mg (Table 1). Diluting the Tris stock 100-fold lowers the buffer capacity by the same factor but leaves the pH unchanged, because adding solvent does not alter the ratio of conjugate base to weak acid, which is what the Henderson–Hasselbalch relation makes the pH depend on. The EDTA content fixes the chelating capacity of the preparation: 1 mM in 100 mL is 0.100 mmol, and since chelation proceeds 1:1 regardless of the charge of the ion, the medium can sequester at most **100 µmol of divalent cations** in total.

Component	Stock	Final	DF	Amount for 100 mL	Role in the medium
Tris-HCl, pH adjusted	1 M	10 mM	100	1.00 mL	holds the pH
Na_2EDTA, pH 8.0	160 mM	1 mM	160	0.625 mL (625 µL)	chelates Ca^{2+} / Mg^{2+}
BSA, powder	solid	0.1 % w/v	–	0.100 g (100 mg)	binds free fatty acids; coats surfaces
Distilled water	–	–	–	to 100 mL	solvent

Table 1. Composition of the 100 mL working buffer. The two stock volumes were combined in a single cylinder and brought to volume together, giving one solution containing both components at their final concentrations.

4.2 The sucrose series

For a 5 mL preparation m = (% w/v × 5)/100, which gives **0.75, 1.15, 1.55, 2.00 and 2.75 g** of sucrose for the 15, 23, 31, 40 and 55 % solutions respectively, 8.20 g of sucrose for the complete series (Table 2). Converting through MW = 342.30 g mol^{-1} shows that the same five solutions span 0.44 to 1.61 M, a useful check that the two concentration units describe the same preparation, and the densities calculated from the apparent specific volume of sucrose run from 1.056 to 1.209 g cm^{-3}. The series therefore brackets the 1.17–1.21 g cm^{-3} at which mitochondria are reported to band in sucrose, with the 40 % solution lying just below that window and the 55 % solution just above it (Figure 1b) — which is the reason the protocol specifies these particular five concentrations rather than a uniformly spaced set.

Sucrose (% w/v)	Sucrose weighed per 5 mL (g)	Concentration (g L^{-1})	Molarity (M)	Calculated density at 20 °C (g cm^{-3})
15	0.75	150	0.438	1.056
23	1.15	230	0.672	1.086
31	1.55	310	0.906	1.117
40	2.00	400	1.169	1.151
55	2.75	550	1.607	1.209

Table 2. The five sucrose solutions, each of 5 mL, prepared in TE + 0.1 % BSA. Molarity and density are calculated, not measured; the density estimate uses the apparent specific volume of sucrose (0.617 cm^3 g^{-1}) and the density of water at 20 °C.

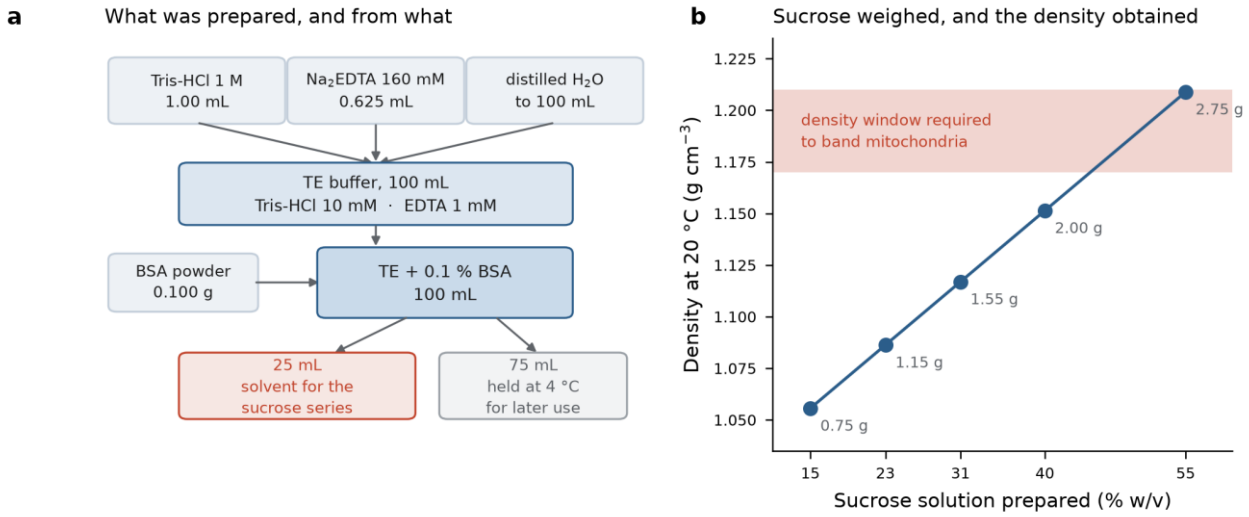


Figure 1. (a) Material balance of the session: the two stocks and the water that make up the 100 mL of TE buffer, the BSA added to it, and the division of the resulting medium between the sucrose series and the volume held in reserve. (b) Mass of sucrose weighed for each 5 mL solution and the density the solution reaches, against the density window the series is designed to bracket.

4.3 Where the uncertainty of these solutions comes from

Since the uncertainty of a balance reading is taken as half its sensitivity, the analytical balance contributes 0.05 mg, which on the smallest mass of the series (0.75 g) is 0.007 % and is negligible; the technical balance contributes 0.005 g, which on the same mass is 0.67 % and is not. Weighing the sucrose on the analytical balance therefore moves the dominant error entirely into the volume: a 5 mL volume set with a micropipette of typical 0.8 % accuracy gives a combined relative uncertainty of about **0.8 %** on the final concentration, essentially independent of which member of the series is considered, whereas setting the same volume in a 10 mL class B graduated cylinder, whose tolerance is of the order of 0.2 mL, would give about 4 % — five times worse, and larger than the spacing between the 15 % and 23 % members of the series. The practical conclusion is that for these preparations the choice of volumetric device, not the choice of balance, sets the quality of the result. No measurements were made on the finished solutions: they were visually clear and colourless, the 55 % solution took appreciably longer to dissolve than the others, and the BSA-containing buffer was obtained free of foam. Density was not verified densitometrically and pH was not re-measured after dilution.

5. Discussion, conclusions and applications

The session delivered 100 mL of Tris-HCl 10 mM / EDTA 1 mM buffer containing 0.1 % w/v BSA, of which 25 mL was consumed as the solvent of five 5 mL sucrose solutions at 15, 23, 31, 40 and 55 % w/v, the remaining 75 mL being held at 4 °C. Judged against the specification set out in Section 1, each requirement is met by an identifiable component: Tris holds the pH, EDTA removes the divalent cations and with them the ion-dependent degradative enzymes, BSA intercepts free fatty acids and passivates the glass and plastic surfaces, and sucrose provides a graded density that brackets the target. The decision to use the buffer, rather than distilled water, as the solvent of the sucrose solutions is what ties the two halves of the exercise together: sucrose dissolved in water would supply density and nothing else, and organelles suspended in it would meet an unbuffered medium containing free Ca^{2+} and Mg^{2+} and no fatty-acid scavenger — they would be exposed to an uncontrolled pH, to nucleases, proteases and phospholipases, and to uncoupling, and the preparation would be compromised before the centrifuge was ever started.

Four limitations follow from the composition and should be carried into the next session. (i) The Tris pK_a of 8.06 shifts by about 0.03 units per degree, so the buffer titrated at 25 °C will read roughly 0.6 units higher at the 4 °C at which it will be used; the pH must be set at the working temperature, and this is one of the commonest errors in a biochemistry laboratory. (ii) The 100 µmol chelating capacity is finite, and because EDTA sequesters Mg^{2+} as well as Ca^{2+} it will also inhibit ATP-dependent enzymes and interfere with nucleotide binding; magnesium must be restored, or the buffer exchanged, before any functional assay, and EDTA-containing buffers are incompatible with immobilised-metal affinity chromatography. (iii) The medium contains 1 mg mL^{-1} of BSA, so protein determinations on anything prepared in it must be read against a buffer-matched blank; moreover the Lowry assay is sensitive to both Tris and EDTA, the two buffer components used here, which makes the bicinchoninic acid assay — far more tolerant of interfering compounds, and about twenty times more sensitive — the appropriate choice. (iv) The sucrose solutions are markedly hyperosmotic, the densest of them at 1.6 osmol L^{-1}, which is intrinsic to the use of sucrose as a density medium and is the reason iso-osmotic alternatives exist.

Beyond this course, the same preparation underlies any procedure that begins by separating subcellular compartments: mitochondrial bioenergetics and respirometry, the detection of cytochrome c release in apoptosis, mitochondrial proteomics and mtDNA analysis, studies of mitochondrial quality control, and the screening of candidate drugs for mitochondrial liability. The immediate application is nearer, however: the solutions prepared and characterised here are the starting material of the next laboratory session, in which they will be used to fractionate a tissue homogenate and to isolate mitochondria. That separation, the centrifugal theory behind it and the fractions it yields are reported separately.

References

- Wilson K., Walker J. *Principles and Techniques of Biochemistry and Molecular Biology*, 8th ed. Cambridge University Press, 2018.
- Good N.E. et al. Hydrogen ion buffers for biological research. *Biochemistry* 1966; 5: 467–477.
- Graham J.M. Isolation of mitochondria from tissues and cells by differential centrifugation. *Curr. Protoc. Cell Biol.* 2001; Unit 3.3.
- Frezza C., Cipolat S., Scorrano L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat. Protoc.* 2007; 2: 287–295.
- Smith P.K. et al. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 1985; 150: 76–85.