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# Stabilisation of Haemoglobin by Maltose: A Comprehensive Ultrasonic, Volumetric, and Compressibility Study

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**Abstract:** ***The stabilisation of haemoglobin (Hb) by maltose in aqueous solution was investigated using density and ultrasonic velocity measurements at five temperatures (303.15–323.15 K) and five concentrations (0.02–0.10 mol kg$^{-1}$). From the experimental data, four acoustico-physical parameters were derived: adiabatic compressibility ($\beta^s$), compressibility lowering ($\Delta\beta^s$), specific acoustic impedance (Z), and relative association (RA). The systematic decrease in $\beta^s$ and the concurrent increase in $\Delta\beta^s$, Z, and RA with rising maltose concentration indicate that maltose strengthens the hydrophobic, electrostatic, and hydrogen-bonding interactions within the haemoglobin structure, thereby impeding denaturation. The results are interpreted in terms of preferential hydration of the protein in the presence of maltose, which amplifies the hydrophobic effect and promotes a compact, folded conformation. These findings provide quantitative insight into the stabilisation mechanism relevant to pharmaceutical formulation, blood substitute development, and food-protein technology.***

**Keywords:** Haemoglobin; Maltose; Adiabatic compressibility; Ultrasonic velocity; Protein stabilisation; Hydrophobic interaction; Preferential hydration

## 1. Introduction

Proteins are thermodynamically metastable macromolecules whose functional integrity depends on maintaining a precisely folded native conformation. Any perturbation, thermal, chemical, or mechanical, can tip the delicate equilibrium towards the denatured state, resulting in loss of biological activity and, in many cases, aggregation. Understanding the molecular forces that govern protein stability in aqueous solution is therefore of fundamental importance in biochemistry, biophysics, and biotechnology (*Romero & Esteso, 2021*; *Dill & Shortle, 1991*).

Sugars and polyols have long been recognised as potent protein stabilisers, both in vivo ,as part of the cellular stress response, and in vitro, where they are used extensively to formulate stable protein therapeutics and enzyme preparations (*Arakawa & Timasheff, 1982*; *Klibanov, 1983*). Despite decades of research, however, the precise molecular mechanism by which a given sugar stabilises a particular protein remains an active area of inquiry. Three non-mutually exclusive mechanisms have been proposed: (i) preferential exclusion of the cosolute from the protein surface (*Arakawa & Timasheff, 1982*), leading to preferential hydration; (ii) direct hydrogen bonding between sugar hydroxyl groups and protein polar residues (*Back et al., 1979*); and (iii) modification of the bulk water structure and its dielectric constant, which in turn modulates electrostatic and hydrophobic interactions (*Akerlof, 1932*; *Patel & Chauhan, 2022*).

Haemoglobin (Hb) is a globular tetrameric protein with a molecular weight of approximately 64.5 kDa. Beyond its central role as an oxygen carrier in erythrocytes, Hb serves as a valuable model system for biophysical studies due to its well-characterised three-dimensional structure, commercial availability in high purity, and relevance to blood-substitute and haemoglobin-based oxygen carrier (HBOC) research (*Zhang et al., 2022*). Maltose (4-O-α-D-glucopyranosyl-D-glucose) is a reducing disaccharide comprising two glucose units linked by an α(1→4) glycosidic bond. Its multiple hydroxyl groups make it an effective hydrogen-bond donor and acceptor, and its capacity to form dense hydrogen-bond networks with water molecules renders it a candidate protein-stabilising agent.

Acoustic and volumetric methods, specifically measurements of ultrasonic velocity and density, offer a non-destructive and highly sensitive probe of solute–solvent and solute–solute interactions in solution (*Chalikian et al., 1993*; *Wadi & Ramasami, 1997*). The adiabatic compressibility ($\beta^s$) derived from these measurements reflects the 'tightness' of the solution structure: a decrease in $\beta^s$ signals increased molecular organisation and stronger intermolecular interactions. Compressibility lowering ($\Delta\beta^s$), specific acoustic impedance (Z), and relative association (RA) provide complementary information on solvation, solute-induced changes in solvent structure, and the degree of molecular association, respectively.

Despite the body of work on sugar–protein interactions, a systematic volumetric and compressibility study of the Hb–maltose–water system across a range of concentrations and physiologically relevant temperatures is absent from the literature (*Banerjee & Bhattacharya, 2021*). The present study addresses this gap by measuring the density and ultrasonic velocity of maltose solutions in a fixed background of haemoglobin at molalities ranging from 0.02 to 0.10 mol kg$^{-1}$ and temperatures from 303.15 to 323.15 K. The derived acoustico-physical parameters are analysed to elucidate the mechanism by which maltose stabilises haemoglobin, with particular emphasis on the role of hydrophobic and electrostatic interactions and the modification of the hydration shell.

## 2. Materials and Methods

### 2.1 Reagents

Haemoglobin (lyophilised powder, purity ≥ 95%) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and used without further purification. Maltose (extra pure grade) was obtained from Qualigens Co. (India). Prior to use, maltose was dried over $P^2O^5$ in a vacuum desiccator for 48 h at room temperature to remove residual moisture. All solutions were prepared with double-distilled water (specific conductance < 2 μS $cm^{-1}$, pH 6.8 ± 0.1) by mass using a Mettler analytical balance (±0.01 mg). A stock haemoglobin solution of fixed concentration was used as the 'solvent' for subsequent preparation of maltose solutions, ensuring that the protein concentration remained constant across all compositions studied.

### 2.2 Density Measurements

Solution densities were measured over the temperature range 303.15–323.15 K at intervals of 5 K using a calibrated Ostwald-Sprengel type pycnometer (volume 8 mL, accuracy ±0.4 mg $mL^{-1}$). The pycnometer was immersed in a thermostatic paraffin oil bath whose temperature was maintained to ±0.1 K by a Julabo temperature controller. Each density value reported is the mean of at least three independent measurements.

### 2.3 Ultrasonic Velocity Measurements

Ultrasonic velocities were measured using a single-frequency (4 MHz) interferometer (Mittal's model F-81) based on the principle of acoustic resonance. The liquid under investigation was placed in a double-walled cell through which thermostated water was circulated to maintain the desired temperature (±0.1 K). A piezoelectric quartz crystal affixed to the base of the cell generated longitudinal ultrasonic waves that were reflected by an adjustable metallic reflector plate. Varying the separation between the crystal and the reflector caused successive resonance maxima in the anode current; the micrometer reading at each maximum was recorded. The ultrasonic wavelength (λ) was determined from the relation $d = n\lambda/2$, where d is the total distance traversed by the micrometer for n maxima. The ultrasonic velocity (U) was then obtained as $U = \nu\lambda$, where $\nu = 4$ MHz is the operating frequency. The experimental accuracy in velocity determination was ±0.3 m $s^{-1}$. All measurements were performed in triplicate and the results averaged.

### 2.4 Computation of Acoustico-Physical Parameters

The adiabatic compressibility ($\beta^s$, $cm^2$ $dyne^{-1}$) was calculated from the Laplace equation (*Jacobson, 1952*):

$$\beta s = 1 / (U^2 \cdot d) \quad ...(1)$$

where U is the ultrasonic velocity (m $s^{-1}$) and d is the density (g $cm^{-3}$). Compressibility lowering ($\Delta\beta^s$) was obtained as the difference between the adiabatic compressibility of the solvent ($\beta^0$) and that of the solution ($\beta^s$) at the same temperature (*Singh et al., 2023*):

$$\Delta\beta s = \beta^o - \beta s \quad ...(2)$$

Specific acoustic impedance (Z, g $cm^{-2}$ $s^{-1}$) was computed as (*Singh et al., 2023*):

$$Z = U \cdot d \quad ...(3)$$

Relative association (RA, dimensionless) was evaluated according to (*Wadi & Ramasami, 1997*):

$$RA = (d / d^o) \cdot (U^o / U)^{(1/3)} \quad ...(4)$$

where $d^o$ and $U^o$ refer to the density and ultrasonic velocity of the solvent (haemoglobin–water) in the absence of maltose.

## 3. Results and Discussion

### 3.1 Density

The densities (ρ, g $cm^{-3}$) of the maltose–haemoglobin–water system measured over the full concentration and temperature range are compiled in Table 1. For all compositions, ρ decreases monotonically with temperature, consistent with the thermal expansion of the solution. Conversely, at any fixed temperature, ρ increases linearly with maltose molality. The addition of 0.10 mol $kg^{-1}$ maltose raises ρ by approximately 0.0068 g $cm^{-3}$ relative to the lowest concentration studied (0.02 mol $kg^{-1}$) at 303.15 K. This behaviour is consistent with the higher molecular mass of maltose (342.3 g $mol^{-1}$) relative to water and with the preferential solvation of the protein surface, which effectively increases the mass density of the solvation shell (*Bhat & Ahluwalia, 1995*; *Romero & Esteso, 2021*).

**Table 1:** Densities ρ (g $cm^{-3}$) of the Maltose–Haemoglobin–Water system as a function of molality and temperature.

| Molality (mol $kg^{-1}$) | 303.15 K | 308.15 K | 313.15 K | 318.15 K | 323.15 K |
|---|---|---|---|---|---|
| 0.02 | 1.0054 | 1.0038 | 1.0022 | 1.0006 | 0.9980 |
| 0.04 | 1.0066 | 1.0050 | 1.0034 | 1.0018 | 1.0002 |
| 0.06 | 1.0086 | 1.0068 | 1.0052 | 1.0034 | 1.0018 |
| 0.08 | 1.0102 | 1.0084 | 1.0068 | 1.0050 | 1.0034 |
| 0.10 | 1.0122 | 1.0104 | 1.0086 | 1.0068 | 1.0050 |

*Values accurate to ±0.4 mg $mL^{-1}$. Background haemoglobin concentration fixed at 1.0 g $dL^{-1}$.*

### 3.2 Ultrasonic Velocity

Table 2 presents the ultrasonic velocities (U, m $s^{-1}$) of the system. Two clear trends emerge: (i) U increases with temperature at all compositions, and (ii) U increases with maltose concentration at all temperatures. The positive temperature dependence of U in protein solutions, which contrasts with the negative dependence observed in pure water above ~74°C , has been attributed to thermal disruption of the hydrogen-bonded water network surrounding the protein, reducing the 'looseness' of the hydration shell and increasing the propagation speed of longitudinal sound waves (*Chalikian et al., 1993*). The concentration dependence is ascribed to strengthened intermolecular interactions upon maltose addition, which raises the elastic modulus of the solution. Nakamura & Ito (2021) reported analogous behaviour in maltose–lysozyme systems, supporting the generality of this observation.

**Table 2:** Ultrasonic velocities U (m $s^{-1}$) of the Maltose–Haemoglobin–Water system.

| Molality (mol $kg^{-1}$) | 303.15 K | 308.15 K | 313.15 K | 318.15 K | 323.15 K |
|---|---|---|---|---|---|
| 0.02 | 1523.4 | 1531.2 | 1536.3 | 1542.9 | 1550.9 |
| 0.04 | 1524.3 | 1532.4 | 1539.2 | 1547.4 | 1552.5 |
| 0.06 | 1526.6 | 1535.0 | 1541.2 | 1549.3 | 1554.6 |
| 0.08 | 1527.1 | 1536.7 | 1542.3 | 1550.7 | 1556.8 |
| 0.10 | 1529.0 | 1539.1 | 1545.0 | 1552.4 | 1559.3 |

*Values accurate to ±0.3 m $s^{-1}$.*

### 3.3 Adiabatic Compressibility

The adiabatic compressibility values ($\beta^s \times 10^{-7}$ $cm^2$ $dyne^{-1}$) listed in Table 3 decrease with both increasing temperature and increasing concentration. The decrease with temperature is interpreted as arising from progressive thermal breaking of solvent clusters, which paradoxically increases the contact between solute molecules and increases the solution rigidity, as reported previously for amino-acid and peptide systems (*Hedwig & Hoiland, 1991*; *Banipal & Sehgal, 1995*). The decrease in $\beta^s$ with maltose concentration, which amounts to ~0.60 × $10^{-9}$ $cm^2$ $dyne^{-1}$ per 0.01 mol $kg^{-1}$ increment at 303.15 K, is attributed to maltose-induced compaction of the haemoglobin structure. As disaccharide molecules accumulate at the solvation layer, they strengthen the hydrogen-bond network through their eight freely-rotating hydroxyl groups, reducing the void volume accessible to compression (*Roberts & Clark, 2023*). This is consistent with the well-established observation that globular proteins possess positive compressibility due to internal cavities, and that any factor that reduces these cavities lowers $\beta^s$ (*Jacobson, 1952*; *Miyahara, 1956*).

**Table 3:** Adiabatic Compressibility βs (×$10^{-7}$ $cm^2$ $dyne^{-1}$) of the Maltose–Haemoglobin–Water system

| Molality (mol $kg^{-1}$) | 303.15 K | 308.15 K | 313.15 K | 318.15 K | 323.15 K |
|---|---|---|---|---|---|
| 0.02 | 4.2858 | 4.2490 | 4.2276 | 4.1982 | 4.1658 |
| 0.04 | 4.2757 | 4.2373 | 4.2066 | 4.1688 | 4.1481 |
| 0.06 | 4.2543 | 4.2154 | 4.1882 | 4.1520 | 4.1303 |
| 0.08 | 4.2448 | 4.1994 | 4.1756 | 4.1379 | 4.1121 |
| 0.10 | 4.2259 | 4.1780 | 4.1514 | 4.1214 | 4.0989 |

*Calculated from Equation (1) using experimental ρ and U values.*

### 3.4 Compressibility Lowering (Δβs)

Table 4 presents the compressibility lowering values ($\Delta\beta^s \times 10^{-7}$ $cm^2$ $dyne^{-1}$). $\Delta\beta^s$ increases monotonically with maltose concentration across all temperatures studied, indicating that maltose consistently reduces the compressibility of the protein solution relative to the protein-only reference. At 303.15 K, $\Delta\beta^s$ increases from 0.0493 at 0.02 mol $kg^{-1}$ to 0.1092 at 0.10 mol $kg^{-1}$, a 2.2-fold increase that scales approximately linearly with concentration. The relationship with temperature is less regular, reflecting the opposing contributions of thermal expansion and solute–solute interactions. The compressibility lowering parameter provides a direct measure of solute–solvent interaction: a larger $\Delta\beta^s$ indicates that the solute causes a greater reduction in the compressibility of the medium, implying stronger solvation (*Wadi & Ramasami, 1997*; *Das & Ghosh, 2022*). The progressive increase in $\Delta\beta^s$ with maltose loading therefore signifies an increasingly rigid solvation shell around haemoglobin, consistent with the preferential hydration model.

**Table 4:** Compressibility Lowering Δβs (×$10^{-7}$ $cm^2$ $dyne^{-1}$) of the Maltose–Haemoglobin–Water system.

| Molality (mol $kg^{-1}$) | 303.15 K | 308.15 K | 313.15 K | 318.15 K | 323.15 K |
|---|---|---|---|---|---|
| 0.02 | 0.0493 | 0.0482 | 0.0363 | 0.0259 | 0.0371 |
| 0.04 | 0.0594 | 0.0600 | 0.0573 | 0.0553 | 0.0548 |
| 0.06 | 0.0778 | 0.0818 | 0.0757 | 0.0722 | 0.0726 |
| 0.08 | 0.0903 | 0.0978 | 0.0883 | 0.0863 | 0.0909 |
| 0.10 | 0.1092 | 0.1192 | 0.1125 | 0.1027 | 0.1040 |

*Calculated from Equation (2): $\Delta\beta s = \beta^o - \beta s$.*

### 3.5 Specific Acoustic Impedance (Z)

The specific acoustic impedance (Z = U · d, g $cm^{-2}$ $s^{-1}$) presented in Table 5 increases with both concentration and temperature. Z is a measure of the resistance of the medium to the propagation of sound; its increase signifies greater molecular cohesion and stronger intermolecular forces (*Tiwari & Srivastava, 2023*). The dual dependence of Z on both thermal energy (via U) and mass density (via d) explains its monotonic rise with temperature even as d falls, since the concurrent rise in U more than compensates for the decrease in d. At any fixed temperature, the increase in Z with maltose concentration reflects the enhanced mechanical rigidity of the solution arising from protein–sugar–water interactions.

**Table 5:** Specific Acoustic Impedance Z (g $cm^{-2}$ $s^{-1}$) of the Maltose–Haemoglobin–Water system

| Molality (mol $kg^{-1}$) | 303.15 K | 308.15 K | 313.15 K | 318.15 K | 323.15 K |
|---|---|---|---|---|---|
| 0.02 | 1531.62 | 1537.01 | 1539.67 | 1543.82 | 1547.79 |
| 0.04 | 1534.36 | 1540.06 | 1544.43 | 1550.18 | 1552.81 |
| 0.06 | 1539.72 | 1545.43 | 1549.21 | 1554.56 | 1557.39 |
| 0.08 | 1542.67 | 1549.66 | 1552.78 | 1558.45 | 1562.09 |
| 0.10 | 1547.65 | 1555.10 | 1558.69 | 1562.95 | 1567.09 |

*Calculated from Equation (3): Z = U · d.*

### 3.6 Relative Association (RA)

Table 6 lists the relative association (RA) values. RA increases with maltose concentration at all temperatures, while its variation with temperature is comparatively small (< 0.001 units across the 20 K range studied). RA values greater than unity at all conditions confirm that the addition of maltose promotes molecular association in the solution. RA is governed by two competing processes: (i) disruption of solvent clusters upon dissolution of the solute (decreasing RA), and (ii) solvation of solute molecules (increasing RA) (*Wadi & Ramasami, 1997*). The fact that RA systematically exceeds 1 and rises with maltose concentration demonstrates that the solvation of maltose and its interaction with the haemoglobin molecule dominate over the breaking of water clusters, thereby promoting a higher degree of molecular organisation. This is further corroborated by the work of Chen et al. (2023) on enzyme–sugar systems, where RA values above unity were correlated with enhanced enzyme stability.

**Table 6:** Relative Association (RA) of the Maltose–Haemoglobin–Water system

| Molality (mol kg$^{-1}$) | 303.15 K | 308.15 K | 313.15 K | 318.15 K | 323.15 K |
|---|---|---|---|---|---|
| 0.02 | 1.0044 | 1.0049 | 1.0058 | 1.0065 | 1.0056 |
| 0.04 | 1.0054 | 1.0058 | 1.0064 | 1.0067 | 1.0074 |
| 0.06 | 1.0068 | 1.0069 | 1.0078 | 1.0079 | 1.0086 |
| 0.08 | 1.008 | 1.0083 | 1.0091 | 1.0092 | 1.0097 |
| 0.10 | 1.0099 | 1.0098 | 1.0103 | 1.0106 | 1.0108 |

*Calculated from Equation (4): RA = (d/d⁰)(U⁰/U)^(1/3).*

### 3.7 Mechanistic Interpretation: Stabilisation of Haemoglobin by Maltose

The convergent evidence from all four acoustico-physical parameters points to a coherent molecular mechanism for maltose-mediated haemoglobin stabilisation. Three inter-related effects contribute:

**Preferential Hydration and Exclusion:** The primary driving force is the preferential exclusion of maltose from the immediate protein surface, which has been established thermodynamically for a wide range of polyhydric cosolutes (*Arakawa & Timasheff, 1982*). Because water molecules solvate the protein surface more strongly than maltose molecules, the protein is effectively 'surrounded' by a water-rich micro-environment. Exclusion of the cosolute from the protein domain raises the chemical potential of the denatured state, which exposes a larger hydrophobic surface area, more than that of the native state, thus thermodynamically favouring the folded conformation. The increasing $\Delta\beta^s$ and RA with maltose concentration provide direct quantitative evidence for the growing extent of this preferential hydration.

**Hydrogen Bonding and Water Structuring:** The multiple hydroxyl groups of maltose compete with protein backbone and side-chain groups for hydrogen bonds with water (*Back et al., 1979*). Rather than binding directly to the protein, which would be stabilising only for the surface-exposed state, maltose hydrogen bonds preferentially to bulk water, forming a more organised, ice-like water network in the vicinity of the protein (*Patel & Chauhan, 2022*). This ordered hydration layer restricts the conformational freedom of the polypeptide chain, limiting unfolding. The increase in RA with concentration is consistent with such progressive water ordering.

**Electrostatic Enhancement and Hydrophobic Compaction:** Sugar solutions possess a lower dielectric constant than pure water (*Akerlof, 1932*; *Das & Ghosh, 2022*), which strengthens electrostatic interactions within the protein. More importantly, the reduced dielectric constant of the maltose–water medium creates a more polar environment at the protein surface, driving non-polar residues towards the protein interior. This amplification of the hydrophobic effect promotes a more compact, denaturation-resistant conformation, as evidenced by the systematic decrease in $\beta^s$ and the increase in Z. These observations are in agreement with the findings of Tiwari & Srivastava (2023) for haemoglobin in trehalose solutions and Roberts & Clark (2023) for various sugars with albumin.

Taken together, these three effects explain why the extent of haemoglobin denaturation is reduced in the presence of maltose: the protein is held in its native conformation by a combination of thermodynamic exclusion forces, a tightly ordered hydration layer, and enhanced internal hydrophobic and electrostatic cohesion. The quantitative relationships between the acoustic parameters and maltose concentration established in this work provide a useful predictive framework for selecting optimal stabiliser concentrations in formulation applications.

## 4. Conclusions

The present study has established, for the first time, a comprehensive set of acoustico-physical parameters, $\beta^s$, $\Delta\beta^s$, Z, and RA, for the maltose–haemoglobin–water system across a physiologically relevant temperature window (303.15–323.15 K) and a practical concentration range (0.02–0.10 mol kg$^{-1}$). The key conclusions are: (i) maltose consistently lowers the adiabatic compressibility of haemoglobin solutions, indicating a more rigid and organised solution structure; (ii) compressibility lowering, specific acoustic impedance, and relative association all increase with maltose concentration, confirming progressive molecular compaction and enhanced solvation; (iii) the stabilisation mechanism operates principally through preferential hydration of haemoglobin, maltose-induced water structuring, and amplification of hydrophobic and electrostatic interactions; and (iv) the magnitude of all effects increases with concentration, suggesting that higher maltose loadings provide greater thermodynamic protection against denaturation, up to the solubility limit.

These findings have practical implications for the design of sugar-based excipients in protein formulations, for the stabilisation of haemoglobin-based oxygen carriers (HBOCs), and for the preservation of blood products. Future work should extend the study to lower temperatures (273–298 K) relevant to cold-chain storage, to higher haemoglobin concentrations mimicking physiological conditions, and to comparative studies with other disaccharides (sucrose, lactose, trehalose) to establish structure–activity relationships.

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