An Isopiestic Comparison Method for Activities

THE ACTIVITIES OF L-SERINE AND L-ARGININE-HYDROCHLORIDE*

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(Received for publication, December 19, 1962)

In establishing the thermodynamic properties of compounds of biological interest, activity coefficients are needed for two common types of calculation. To compute the standard free energy of formation in solution, the activity of the solute in the saturated solution is required; to calculate standard free energy changes from equilibrium constants, activities rather than stoichiometric concentrations are necessary. In our studies of amino acids, we have found it convenient to employ the isopiestic comparison method for measuring activities introduced by Sinclair (1), following a suggestion from Bousfield and Bousfield (2). The method was refined by Robinson and Sinclair (3) and has been widely applied to inorganic salts, notably by Robinson et al. For extensive references, see Robinson and Stokes (4) or Harned and Owen (5). Several amino acids were studied by Smith and Smith (6-9) and by Richards (10). Recently, an attempt has been made to apply the method at temperatures up to 100° (11).

In most of the work cited above, the technique employed consists, in essence, of enclosing two or more shallow metal dishes, each containing approximately 2 ml of solution, in a large evacuated glass or metal container. To facilitate heat transfer between the dishes, they are sunk into a massive block of copper. The entire assembly is immersed in a constant temperature bath and shaken. After 2 to 8 days, the vacuum is broken and the dishes are removed and weighed.

Several difficulties beset the method outlined above. (a) Thermal contact between the dishes and the copper block may be poor, requiring the interposing of extra solutions. (b) If extra solution is added to aid heat conduction, the dishes must be washed before weighing. (c) The solution may boil or gas bubbles break during evacuation of the vessel. (d) A large and cumbersome assembly must be shaken. (e) The diffusion paths are long. (f) Fog may form when the vacuum is broken. (g) Sealing the dishes during weighing is difficult. The last two difficulties do not occur in the method of Soldano et al. (11), who weigh the dishes inside the evacuated space, but the required apparatus is complicated and not readily available.

We have found it possible to overcome most of these difficulties and to devise a method requiring only small amounts of material. When specific gravity gradient tubes (12) are used to measure concentrations of solutions, the solutions to be compared may be put on the same piece of metal rather than in separate containers. Temperature equality is therefore easier to obtain. The small amounts of solution required permit droplets to be placed close together in a small gas space. As a result, the isopiestic condition is achieved in a shorter time and shaking is not necessary. Because the essential assembly is small, several separate comparisons can be made simultaneously in a single constant temperature bath.

We present here our results for L-arginine·HCl and L-serine compared with KCl and sucrose, respectively. DL-Serine was studied by Smith and Smith (9). L-Arginine·HCl has not been investigated previously.

EXPERIMENTAL PROCEDURE

Materials—The basic standard employed was a sample of sucrose obtained from the United States Bureau of Standards (Standard Sample 17, lot No. 6004). A large sample of sucrose was reserved for these studies and compared over a broad range of concentration by our isopiestic method with the standard sample. No differences in isopiestic molalities (±0.01%) were detected.

A large sample of KCl was also reserved for these studies and compared with the standard sucrose sample by the isopiestic method. The relative values of the osmotic coefficients agreed within the limits of experimental error (< 0.1%) with those given by Robinson and Stokes (4).

The amino acids used were provided by the late Jesse P. Greenstein. They met the same criteria of purity previously described (12) for glycine and L-alanine and came from the same batches of materials used in this laboratory for heat capacity and solubility studies.

The distilled water used was passed through a Barnstead Bantam demineralizer and showed < 0.01 p.p.m. as NaCl on a conductivity meter. The solutes were dried under vacuum in a system trapped with liquid nitrogen. Molecular weights used were: KCl, 74.56; sucrose, 342.3; L-arginine·HCl, 210.7; L-serine, 105.1.

Apparatus—The device in which the solutions became isopiestic is shown in Fig. 1. The silver disk (A) was 4.7 cm in diameter and 0.64 cm thick. Into this, four symmetrically disposed depressions (B) 1.4 cm in diameter and 0.32 cm deep were milled. Then the disk and depressions were polished. The enclosing dish (C) and its lid (D) were spun from aluminum. To improve closeness of fit, the 0.64-cm flange of the dish and the overlying portion of the lid were turned on a lathe. The dish and lid were plated first with copper and finally with gold.1 However, pinholes in the gold plating usually could be detected by putting

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1 These dishes were originally made for the established isopiestic method and adapted for the present purpose.
and was constant to +0.001" in the absence of gross failure. A thermometer certified by the United States Bureau of Standards was used. All parts in position, the gas space was approximately 17 ml.

The Pyrex glass stopcock (H) had a 1-mm bore and was cemented into a brass well (I) with Apiezon W cement to form a vacuum seal and to provide mechanical support. With a loose fit, the only sure metal-to-metal contact being around the bottom section (F) received the dish and its lid with relatively rough beading of the flange of the dish. The "0" ring (G) employed was of Buna N rubber, 9.8 cm outside diameter and 8.9 cm inside diameter. The Pyrex glass stopcock (H) had a 1-mm bore and was cemented into a brass well (I) with Apiezon W cement to form a vacuum seal and to provide mechanical support. With all parts in position, the gas space was approximately 17 ml. Eight such assemblies were available, thus permitting several comparisons to be conducted simultaneously.

Two identical water-filled constant temperature baths were constructed. Their temperature was set at 25.00 ± 0.01οC with a thermometer certified by the United States Bureau of Standards and was constant to ±0.001οC in the absence of gross failure. Into each a copper box (22 × 22 × 22 cm), which could accommodate the four gradient tubes shown in Fig. 1, was lowered to a depth of 21 cm. It was covered by a triple-walled copper lid, all three walls of which dipped into the water bath. Except for slight transient differences, caused by introduction or removal of contents, box temperature equaled bath temperature.

A bank of nine gradient tubes (12) was immersed in a water bath with plate glass walls. The water in the bath was topped by approximately 1 cm of paraffin to prevent evaporation, and the gradient tubes were closed with polyethylene stoppers. The tubes contained ligroin-monobromobenzene mixtures, ranging in specific gravity from 0.99 to 1.34, with individual gradients adjusted so as to separate molalities differing by 10% by at least 1 cm. Each ligroin-monobromobenzene mixture was shaken with distilled water or a sucrose solution of corresponding specific gravity. Since the activity of water thus attained could not be equal to that of all solutions which might be put into a given gradient tube, relatively large droplets (4 to 6 cu mm) were used to reduce the exchange between droplets and the gradient tube mixture. To compensate for any such exchanges, the standards employed were known molalities of the same substance.

Droplet positions were ascertained with the aid of one of two devices. One was a cathetometer with an ocular micrometer in the telescope. The telescope-to-droplet distance was adjusted so that the interval between standard droplets would represent approximately 100 ocular divisions. The other was a traveling microscope that had a scale readable to 0.001 mm and reproducible to 0.01 mm. With either device, estimates of droplet position were 10-fold more accurate than were justified by knowledge of the molalities of standard solutions (±0.1%). When the 4- to 6-cu mm drops were used, any exchanges that occurred between them and the gradient tube fluid led only to alterations in absolute position. Relative positions, i.e. the derived molalities of unknowns, were unaffected.

Solutions were made in silicone-coated serum vials with rubber caps compressed by a crimped aluminum seal. The vials were stored at 1οC when not required for withdrawal of samples. Following repeated (20 times) puncture of the rubber caps by a 24-gauge needle, no weight loss could be detected in a sealed vial over a period of several days. Samples for loading the silver blocks or for comparisons of specific gravities were withdrawn with 0.5-ml or 1-ml tuberculin syringes tipped with 24-gauge stainless steel needles. When the samples were to be used for specific gravity comparisons, the needles were immediately covered with ligroin in a tube (2 × 20 mm).

All apparatus was housed and all manipulations were conducted in a temperature-controlled room (24οC ± 0.5οC).

Preparation and Incubation of Samples—A solution of the test substance and one of the reference substance were prepared. Molalities were chosen so that their ratio would be close to (usually within 1% of) that expected at equilibrium. At least 50 mg of solute and at least 2 g of water were used; each was weighed to ±0.05 mg. The chief burden of error was therefore put on weight of the solute.

All parts of the assembly shown in Fig. 1 were cleaned, rinsed with distilled water, and dried in air. The "O" ring, its groove, and the overlying segment of the brass block were lightly greased with Lubrisol. In experiments with l-serine, the silver disk was untreated. In experiments with l-arginine-HCl, the silver disk was covered with a light film of paraffin (m.p. 56-58οC) to prevent corrosion.

Samples of reference and test solutions were withdrawn from the serum vials and aliquots were put into the four depressions in the silver disk. The drops of a given substance were in diametrically opposed depressions, thus producing a radially symmetrical diffusion situation. In experiments with l-serine versus sucrose, the droplets had a volume of 0.2 ml and spread readily on the bare silver block. In experiments with l-arginine-HCl versus KCl, it was necessary to use smaller droplets, 0.1 to 0.15 ml, because the paraffin coating prevented spreading of the droplets and they otherwise would have made contact with the lid. Placing the droplets in either case required less than 1 minute.

The lid of the gold-plated dish was immediately put in place, the top segment of the brass block was mounted and the six 8/32 machine screws were tightened sufficiently to seal the "O" ring. Over a period of 15 minutes, the gas space was evacuated by slow leakage through a stopcock to a 500 ml flask (containing water) which was periodically evacuated with a water pump. Barometric pressure was reduced to P<sub>H2O</sub> + 10 mm, which caused 2 to 5% of the water to evaporate from the test droplets. If the original vacuum was not maintained throughout the experiment, the subsequent analyses were not made.

In control experiments, it was shown that sucrose solutions differing by 10% in molality became identical (±0.01%) within the following time periods: 48 hours at a molality ≥1.0; 96 hours between them and the gradient tube fluid led only to alterations in absolute position. Relative positions, i.e. the derived molalities of unknowns, were unaffected.

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at 0.5 molal; 1 week at 0.1 molal. These or longer periods were employed even though solutions used in experiments originally differed by only 1% from the isopiestic ratio.

Measurement of Molality—After appropriate periods (48 hours to 1 week) in the constant temperature box, the assemblies were removed, the pressure checked, and the vacuum broken with air slowly admitted through a column of dry, indicating silica gel. The screws were released, and the upper brass block was removed.

Aliquots of the solutions on the silver block were removed with Pyrex pipettes, the tips of which were immediately covered with a lignin-filled tube. The entire sampling procedure required only 20 to 30 seconds. The gold-plated lid and the entire interior of the system were inspected for evidence of splashing or fogging.

A ligroin-filled tube, the entire sampling procedure required 2 to 3 minutes had elapsed, the positions of the droplets were read with one of the cathetometers.

Analysis of Data—We have utilized the osmotic coefficients, \( \phi \), of sucrose and KCl given by Robinson and Stokes (4). No important difference in activity coefficients is produced if the values of sucrose and KCl given by Robinson and Stokes (4). No important difference in activity coefficients is produced if the values of each pair of duplicate solutions agree within 0.1% in molality.

The specific gravities of reference and test solutions differed so widely that even minor contamination of one by the other would have been detected.

Samples of known molality were withdrawn from the bottles into the syringes mentioned, and droplets were introduced into the gradient tubes in the following order: higher molality; unknowns; lower molality. Uefore putting droplets into the gradient tubes in the following order: higher molality; unknowns; lower molality. In the constant temperature box, the assemblies were removed, the pressure checked, and the vacuum broken with air slowly admitted through a column of dry, indicating silica gel. The screws were released, and the upper brass block was removed.

Aliquots of the solutions on the silver block were removed with Pyrex pipettes, the tips of which were immediately covered with a lignin-filled tube. The entire sampling procedure required only 20 to 30 seconds. The gold-plated lid and the entire interior of the system were inspected for evidence of splashing or fogging. No experiments were accepted in which readily detectable splashing or fogging occurred. As a further guard against splashing of one solution into another, we required that the specific gravities of each pair of duplicate solutions agree within 0.1% in molality. The specific gravities of reference and test solutions differed so widely that even minor contamination of one by the other would have been detected.

Samples of known molality were withdrawn from the bottles into the syringes mentioned, and droplets were introduced into the gradient tubes in the following order: higher molality; unknowns; lower molality. Before putting droplets into the gradient tubes, three or four droplets were shed into a beaker of lignin next to the gradient tube to purge the pipette tip. After 3 minutes had elapsed, the positions of the droplets were read with one of the cathetometers.

Analysis of Data—We have utilized the osmotic coefficients, \( \phi \), of sucrose and KCl given by Robinson and Stokes (4). No important difference in activity coefficients is produced if the values given by Harned and Owen (5) are employed. Our notations and definitions of terms have also been adapted from Harned and Owen (5). Large scale plots of \( m_1/m_2 \) against \( m_1 \) were prepared in which \( m_1 \) = molality of the reference substance (sucrose or KCl) and \( m_2 \) = molality of the test substance. From the curves drawn through the experimental points, values of \( m_1/m_2 \) were read at appropriate intervals and treated as follows:

(a) For L-serine versus sucrose, values of \( \phi_2 \) were obtained from the relation

\[ \phi_2 = \frac{m_1}{m_2} \phi_r \]

and the value of

\[ -\ln \gamma_2 = - (1 - \phi_2) - \int_0^{m_1} \left( \frac{1 - \phi_2}{m_2} \right) dm_2 \]

was determined by summation of smoothed tabular values at intervals of 0.1 in \( m_1 \).

(b) For L-arginine-HCl versus KCl, plots of \( 1 - \phi_2/m_2 \) were

Following Harned and Owen (5), we define the osmotic coefficients, \( \phi \), as

\[ -1000 \frac{\nu_{M_1} \ln p/p_0}{\nu_{M_1} M_1 RT} \int_0^{p} \alpha dp \]

where \( \nu \) = the number of particles furnished by the solute, \( M_1 \) = the molecular weight of the solute, \( M_1 \) = stoichiometric molality of the solvent, \( p_0 \) = vapor pressure of the pure solvent, and \( p \) = vapor pressure of the solution. The second member, involving \( \alpha \) (a complex term), is customarily dropped as being smaller than experimental errors.

\[ \ln \gamma_2 = - (1 - \phi_2) - 2 \int_0^{m_1} \left( \frac{1 - \phi_2}{m_2} \right) dm_2 \]

The curve was extrapolated to zero concentration with an intercept of \( 1 - \phi_2/m_2 \) = 0.3738 on the assumption that the Debye-Huckel theory applies. L-Arginine-HCl was assumed to behave as a 1:1 electrolyte, an assumption justified, apparently, by the approach of \( m_1/m_2 \) to unity at low concentrations. Intervals of 0.1 in \( m_1 \) were used except in regions of extreme curvature where the intervals were appropriately reduced.

RESULTS

The solutions found to be isopiestic are recorded in Table I. Accuracy beyond 0.2% is not implied in spite of specific figures

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tbody>
<tr>
<td>Molalities of isopiestic solutions of sucrose versus L-serine and KCl versus L-arginine-HCl at 25°</td>
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<tr>
<td>Values are in moles of solute per 1000 g of H2O.</td>
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<tr>
<td>Succrose</td>
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<tr>
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<table>
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<tr>
<th>TABLE II</th>
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<tr>
<td>Activity coefficients of L-serine and L-arginine-HCl at various molalities at 25°</td>
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<tr>
<td>Values are in moles of solute per 1000 g of H2O.</td>
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<tr>
<td>Molality</td>
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<td>0.1</td>
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<td>0.2</td>
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<tr>
<td>4.02</td>
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<td>4.06*</td>
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* Saturated solution.
When analyzed in the manner outlined previously, these data lead to the activity coefficients listed in Table II. The value of \((\partial \ln \gamma)/\partial (\ln m)\) for L-arginine·HCl is \(-0.25\) at 4.00 molal and that for L-serine is \(-0.25\) at 4.02 molal (i.e. at saturation concentration for these substances).

DISCUSSION

Our results confirm the finding of Smith and Smith (9) that the presence of the hydroxyl group in serine lowers its activity in comparison with amino acids having unsubstituted alkyl \(R\)-groups. Their experimental points for \(dL\)-serine satisfactorily fit our curve which, because of the greater solubility of L-serine, extends to higher molalities. In early experiments conducted to establish the adequacy of the present method, we compared glytine and sucrose solutions. The molal ratios obtained agreed satisfactorily with those previously reported (6, 10). We have reevaluated the data (6-9) for various monoamino-monocarboxylic amino acids in terms of the more recent values for the osmotic coefficient of sucrose (4). This leads to intercepts of \(1 - \alpha/m\) plots at approximately 0.13 instead of 0.10 (14). The effect of the newer values on the activity coefficients for the amino acids in saturated solutions is, however, negligible.

Scatchard (15) emphasizes the importance of complete removal of air from systems in which isopiestic equilibria are sought. This evacuation is required in systems where heat transfer is barely sufficient, but the improved heat conduction and small gas space in our system permitted reasonably short incubation periods without drastic evacuation. We were thus able to avoid the splashing that accompanies bursting of bubbles formed in solutions in high vacuum.

More precise values of isopiestic ratios could be obtained through a better knowledge of the molalities of standard solutions. This can readily be achieved through more precise weighing of solutes. That we did not do so reflects our early disenchantment with the method, from which we did not recover in time to improve our procedure.

SUMMARY

1. A modified isopiestic comparison method for measuring the activities of solutes is described.
2. Specific gravity gradient tubes are used for measurement of molalities. This permits placing small droplets of solutions to be compared on a single block of silver and enclosing them in a small gas space. No shaking is required.
3. Results of comparisons of L-arginine·HCl with KCl and of L-serine with sucrose are presented. Activity coefficients at 25° at various concentrations, including saturated solutions, are given.

REFERENCES

An Isopiestic Comparison Method for Activities: THE ACTIVITIES OF 1-SERINE AND 1-ARGININE·HYDROCHLORIDE
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