The Interaction of Proline and Other Imino-acids with *p*-Benzoquinone

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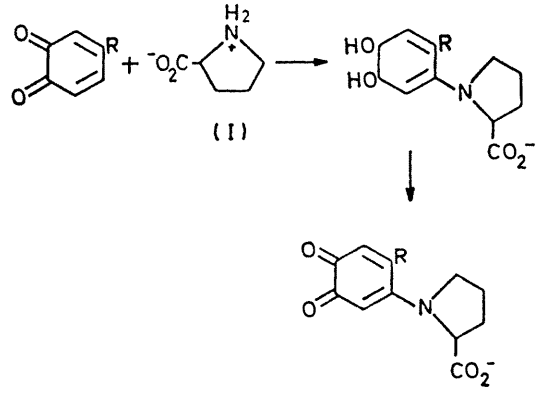
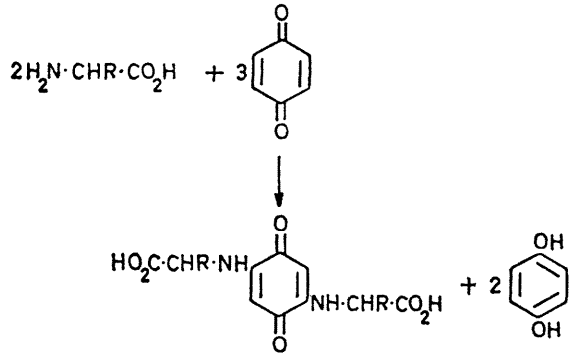
The interaction between proline and other imino-acids with *p*-benzoquinone has been studied spectrophotometrically and found to be due to the initial formation of charge-transfer complexes displaying the characteristic charge-transfer band, contrary to the views of earlier workers. Kinetic and thermodynamic parameters have been evaluated for the interactions.

SEVERAL studies have been carried out on the interaction of amino- and imino-acids with benzoquinones.[1-3] Most were, however, interpreted at a time when the existence



SCHEME 1

of weak complexes, particularly charge-transfer complexes was not yet recognised. Kendall and Jackson [2] have reported complexes formed between imino-acids and both catechol and catecholamine in oxidations catalysed by tyrosinase, and have suggested a mechanism involving an addition product (see Scheme 1). Great difficulty was experinced in isolating the complex as a solid; this was attributed to its instability. A slightly different mechanism has been suggested by Michalek and Szarkowska [3] (Scheme 2).



SCHEME 2

Recently, however, it has been shown that the interaction of amino-acids and proline (I) with tetrachloro-*p*-benzoquinone results in the formation of a 1 : 1 n–π charge-transfer complex of the quinhydrone type, the

[1] H. Beevers and W. O. James, *Biochem. J.*, 1948, **43**, 636.
[2] L. P. Kendall and H. Jackson, *Biochem. J.*, 1949, **44**, 477.
[3] H. Michalek and L. Szarkowska, *Acta Biochem. Polon.*, 1959, **6**, 399.

acids being in the neutral amino-form.[4] *p*-Benzoquinone is the only well-known charge acceptor forming charge transfer complexes, which is both water soluble and of biological interest. It was therefore decided to re-examine the interaction of some imino-acids with *p*-benzoquinone to see whether the original interpretations are valid and to evaluate rate constants and thermodynamic data.

EXPERIMENTAL

Absorption spectra were recorded on a Unicam SP 700 spectrophotometer using 1-cm fused-silica cells. The cell compartments were thermostatically controlled to $\pm\frac{1}{2}°$. An attached timing device was used to obtain plots of transmission *vs.* time for a given wavenumber. All calculations were carried out on an English Electric KDF9 computer.

Materials.—The brownish *p*-benzoquinone (B.D.H.) was purified by microsublimation under nitrogen along a temperature gradient to give a yellow solid. Proline and hydroxyproline (both B.D.H.) were chromatographically

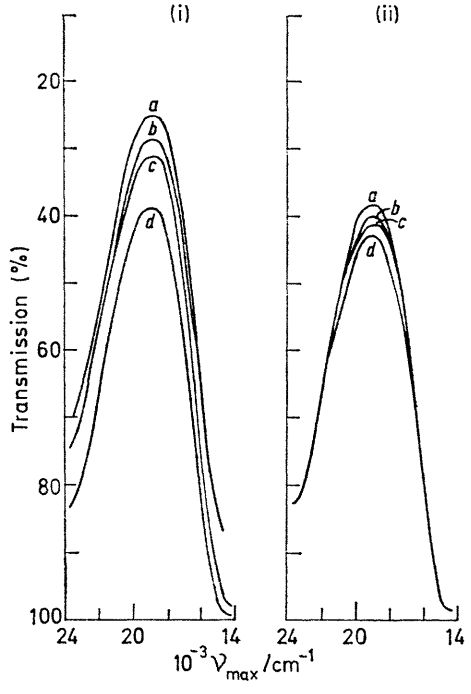


FIGURE 1 (i) Absorption spectra of proline–*p*-benzoquinone mixtures in phosphate buffer pH at 25°, with proline concentration: (*a*) 4×10^{-1}M; (*b*) 10^{-1}M; (*c*) 7×10^{-2}M; (*d*) $2{\cdot}25 \times 10^{-2}$M, and *p*-benzoquinone concentration constant at 2×10^{-4}M. (ii) Effect of temperature on the absorption spectrum of the proline ($2{\cdot}25 \times 10^{-2}$M)–*p*-benzoquinone ($2 \times 10^{-4}$M) mixture in pH 7 buffer at (*a*) 25, (*b*) 35, (*c*) 45, and (*d*) 55 °C

homogeneous. Propylglycine was obtained from Sigma Chemical Co. L-Thiazolidine-4-carboxylic acid and L-azetidine-2-carboxylic acid were obtained from Calbiochem. Ltd. Phosphate buffer, pH 7, was prepared by adding, in solid form, the equivalent of 29·6 ml l^{-1} of N-sodium hydroxide to 0·05N-potassium dihydrogen phosphate. Buffers

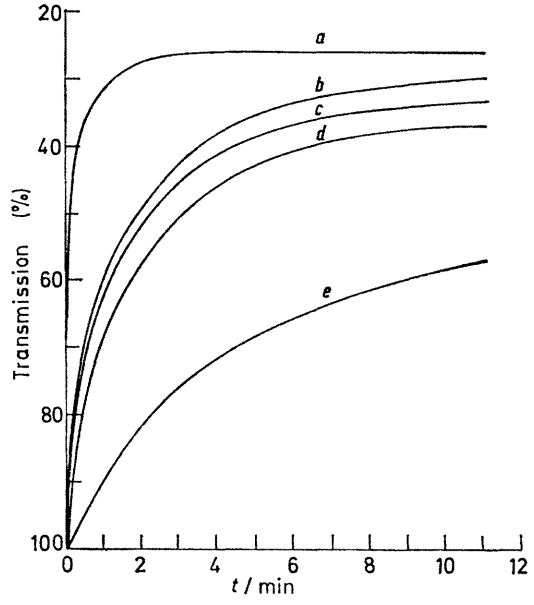


FIGURE 2 Development of absorption spectrum of proline–*p*-benzoquinone with respect to time as function of proline concentration: (*a*) 4×10^{-1}M; (*b*) 10^{-1}M; (*c*) 7×10^{-2}M; (*d*) 4×10^{-2}M; (*e*) $1{\cdot}5 \times 10^{-2}$M. All with 2×10^{-4}M-*p*-benzoquinone in phosphate buffer pH 7 at 25 °C

at other pHs were obtained by adjusting the amount of sodium hydroxide. Deionised water was used throughout.

RESULTS AND DISCUSSION

The addition of proline to *p*-benzoquinone in phosphate buffer at pH 7 caused the appearance of a new spectral band at 18,800 cm^{-1} (see Figure 1). The solutions are purple-red, as described by Kendall and Jackson.[2] A study of the growth of the peak with time shows that the reaction is first order with respect to both compounds and second order overall, indicating a 1 : 1 interaction and is both temperature reversible (Figure 1) and dilution reversible as shown in its excellent agreement with the Benesi–Hildebrand equation.[5]

Apparent kinetic data has been evaluated from the spectral behaviour exemplified in Figures 2 and 3 and by assuming the mechanism [6] [equation (1)] and deriving

$$\text{proline} + p\text{-benzoquinone} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{complex} \qquad (1)$$

$K_c = k_1/k_2$ from equilibrium conditions by the Benesi–Hildebrand equation.[5] Arrhenius plots of the rate constants and equilibrium coefficients (see Table 1) gave the thermodynamic data (see Table 2).

The effect of pH on the rate constants is also shown in Table 1 and it can be seen that there is a very marked increase in forward rate constant with increasing pH.

[4] M. A. Slifkin and R. H. Walmsley, *Experientia*, 1969, **25**, 930.

[5] H. A. Benesi and J. H. Hildebrand, *J. Amer. Chem. Soc.*, 1949, **71**, 2703.

[6] M. A. Slifkin and J. G. Heathcote, *Spectrochim. Acta*, 1969, **23A**, 2893.

This suggests that, contrary to the views of earlier workers,[2,3] the active reactant is the negative ion (*i.e.*, the NH form), similar to other amino- and imino-acid

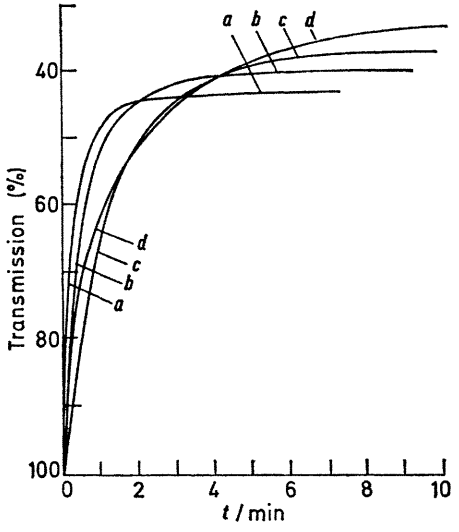


FIGURE 3 Effect of temperature on the development of the proline–*p*-benzoquinone absorption bands at 18,800 cm^{-1} and (*a*) 55, (*b*) 45, (*c*) 35, and (*d*) 25 °C; proline concentration, 7×10^{-2}M, *p*-benzoquinone concentration, 2×10^{-4}M

charge-transfer complexes.[7] Consequently the various parameters have been re-evaluated where possible using to obtain the various data of Tables 1—3 are outlined in the Appendix).

The corrected enthalpy of dissociation, while being relatively high compared with the corresponding enthalpies of organic charge-transfer complexes which are usually not more than *ca.* 12 kcal mol^{-1},[10] is of the same order as that of the complexes of amino-acids with other acceptors when corrected according to equation (2),[7] and much lower than the dissociation enthalpies of covalent bonds. The corrected entropy change suggests that there is considerable ordering in the complex, due to either ordering of the solvent around the complex or a specific orientation of the complex; probably both, since charge-transfer complexes are characterised by the components having a specific orientation with respect to each other, as well as having larger dipole moments than the components.[10]

One interesting result is that for these complexes the corrected forward rate constants are all of the same order even at different temperatures and pHs, and the major factor in deciding the equilibrium constants is the backward rate constant. Consequently even in situations where K' is unknown, k_2 (which is evaluated independently of K') can still be used to give a measure of the strength of the interaction.

The outstanding characteristic of charge-transfer complexes is the charge-transfer transition with its associated band.[10] Briegleb [10] has shown empirically that this band conforms to certain criteria. In Table 3 are listed the parameters from the absorption spectrum of the proline–*p*-benzoquinone complex, together with those of Briegleb,[10] and are seen to be in very good agreement.

TABLE 1

Apparent and corrected rate and equilibrium constants for the proline–*p*-benzoquinone complex [a]

	k_1/l mol^{-1} min^{-1}			K_c/l mol^{-1} [b]		
pH	Apparent	Corrected	k_2/min^{-1}	Apparent	Corrected	θ_c/°C
7	$5{\cdot}88 \times 10^{-6}$	$2{\cdot}57 \times 10^{-2}$	$6{\cdot}23 \times 10^{-7}$	9·45	$4{\cdot}12 \times 10^{4}$	25
7	$7{\cdot}31 \times 10^{-6}$	$1{\cdot}85 \times 10^{-2}$	$1{\cdot}21 \times 10^{-6}$	6·04	$1{\cdot}53 \times 10^{4}$	35
7	$1{\cdot}49 \times 10^{-5}$	$2{\cdot}15 \times 10^{-2}$	$4{\cdot}69 \times 10^{-6}$	3·18	$4{\cdot}58 \times 10^{3}$	45
7	$2{\cdot}59 \times 10^{-5}$	$2{\cdot}36 \times 10^{-2}$	$1{\cdot}41 \times 10^{-5}$	1·84	$1{\cdot}67 \times 10^{3}$	55
6	$7{\cdot}27 \times 10^{-5}$	$3{\cdot}17 \times 10^{-2}$	$2{\cdot}25 \times 10^{-8}$	32·3	$1{\cdot}41 \times 10^{6}$	25
8	$2{\cdot}84 \times 10^{-5}$	$1{\cdot}24 \times 10^{-2}$	$1{\cdot}04 \times 10^{-5}$	2·73	$1{\cdot}21 \times 10^{3}$	25

[a] In phosphate buffer. [b] $K_c = k_1/k_2$.

TABLE 2

Apparent and corrected thermodynamic data for the proline–*p*-benzoquinone complex [a]

Direction	ΔH^*	$\Delta H°$	E_A [b]	ΔS^*	$\Delta S°$	ΔG^*	$\Delta G°$
Forward	9·3 (−1·0)		9·9	−59·6 (−45·5)		27 (12·5)	
		−10·6 (−20·9)			−31·0 (−16·9)		−1·4 (−15·9)
Backward	19·9		20·5	−28·6		28·4	

[a] * denotes parameters for one direction. E_A Is the energy of activation, ΔH is the enthalpy change, ΔS is the entropy change, ΔG is the free energy change, and ° denotes equilibrium values. All parameters are in kcal mol^{-1} except entropy which is in cal K^{-1} mol^{-1}, and are mean values over 25—55°. All forward and equilibrium parameters are apparent except those in parentheses which are corrected.

pK' values from ref. 8 and the free energy, enthalpy, and entropy of dissociation of proline from ref. 9 according to the mechanism in equation (2). (The methods used

$$\text{proline}\,(NH_2^+) \overset{K'}{\rightleftharpoons} \text{proline}\,(NH) + p\text{-benzoquinone} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{complex} \quad (2)$$

[7] M. A. Slifkin, 'Charge Transfer Interactions of Biomolecules,' Academic Press, London, 1971, ch. 3.

[8] C. Long, 'Biochemist's Handbook,' Spon, London, 1962.

[9] P. K. Smith, A. T. Gorham, and E. R. B. Smith, *J. Biol. Chem.*, 1942, **144**, 737.

[10] G. Briegleb, 'Elektron-Donator-Acceptor-Complexe,' Springer, Berlin, 1961.

The interaction observed between proline and *p*-benzoquinone is the formation of a charge-transfer complex of an n–π type, with proline, the donor, in its NH form, the donated electron being one of the n-(lone-pair)

TABLE 3

Characteristics of the absorption band of the proline–*p*-benzoquinone complex

Absorbance	Wavenumber (cm^{-1})
Maximum, $\nu_{max.}$	18,900
Half-maximum, ν_H	21,800
ν_L	16,800

$\nu_H - \nu_L = 2{\cdot}38\ (\nu_{max.} - \nu_L)$,
$\nu_{max.} - \nu_L = 0{\cdot}111\ \nu_{max.}$

Briegleb's criteria

$\nu_H - \nu_L = 2{\cdot}4\ (\nu_{max.} - \nu_L)$,
$\nu_{max.} - \nu_L = 0{\cdot}104\ \nu_{max.}$

TABLE 4

Effect of acetone on the peak absorbance position and apparent rate constants of the proline–*p*-benzoquinone complex at 25 °C

$\nu_{max.}$/ cm^{-1}	k_1/ l mol^{-1} min^{-1}	k_2/ min^{-1}	K_c/ l mol^{-1}	% Acetone added to phosphate buffer (v/v)
19,000	$1{\cdot}5 \times 10^{-5}$	$5{\cdot}42 \times 10^{-6}$	2·78	20
19,000	$2{\cdot}45 \times 10^{-5}$	$1{\cdot}40 \times 10^{-5}$	1·65	40

electrons on the imino-nitrogen atom. This is in marked contradiction with previous theories.[2,3]

The effect of two different solvents on the interaction has been studied. Galzigna [11] has suggested that the mixture dioxan–trisbuffer [0·1M-tris(hydroxymethyl)-methylammonium chloride] (9 : 1) at pH 7 is a suitable solvent for biochemical studies as it has the same conductivity and oxidative properties as whole-brain homogenates. We have been unable to detect any difference between this solvent and the phosphate buffer.

Conversely, acetone has a marked effect on the interaction. There is a strong increase in the apparent forward reaction rate, but an overall decrease in the apparent equilibrium constant (see Table 4). In addition the charge-transfer transition is bathochromically shifted. As acetone is less polar than the water it is displacing, this will inhibit to some extent the amount of charge transfer. In addition as there will be less ordering of the solvent around the molecules, it will be easier for them to approach each other, thus explaining the increase in forward reaction rate. The blue shift can be explained in two equivalent ways. The lowering of the strength of the interaction will cause a blue shift of the charge-transfer transition. Alternatively, in the less polar solvent there will be a decrease in the stability of the excited state of the complex giving rise to a blue shift.

In acetone, hydroquinone formation (detected spectrophotometrically) proceeds faster than in phosphate buffer in the dark. We have found that solutions of *p*-benzoquinone alone in acetone are reduced slowly to hydroquinone and it is likely that the interaction in acetone is independent of complex formation. Hydroquinone is formed very rapidly in irradiated solutions and is presumably a photoproduct.

Hydroquinone production could only be observed in proline–*p*-benzoquinone mixtures in pH 7 phosphate buffer at 55° and then only after a period of several hours. Consequently it is permissible to ignore hydroquinone production in evaluating the kinetics of the formation of the complex. Furthermore it shows that the presence of the proline inhibits hydroquinone production rather than being responsible for it as suggested by previous workers.[2,3]

The interaction of some other imino-acids with *p*-benzoquinone in phosphate buffer at pH 7 has been studied. Table 5 lists the apparent rate constants and positions of the charge-transfer absorption. Some of the complexes are unstable and on standing decompose primarily to hydroquinone. This situation has been analysed using the reaction scheme in equation (3).

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (A:B) \xrightarrow{k_3} C \qquad (3)$$

Where appropriate, values of k_3 are given in Table 5 and it can be seen that these are comparatively small, of the order of 10^{-7} min^{-1}.

TABLE 5

Peak positions and rate and equilibrium constants for the interaction of imino-acids with *p*-benzoquinone in phosphate buffer pH 7 at 25° [a]

Imino-acid	$\nu_{max.}$/cm^{-1}	$10^6 k_1$/l mol^{-1} min^{-1}	$10k_2$/min^{-1}	$10^5 k_3$/min^{-1}	K_c/l mol^{-1}
Proline	18,800	5·88 [b]	6·23		9·45 [d]
Hydroxyproline	19,200	7·93 [c]	9·89		8·02 [e]
Prolylglycine	19,400	5·11	15·3	8·88	3·34
L-Thiazolidine-4-carboxylic acid	18,800	6·69	18·7	9·68	3·58
L-Azetidine-2-carboxylic acid	18,800	9·45	21·4	9·56	4·42

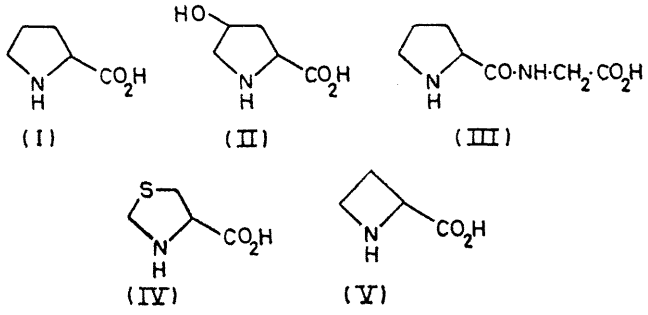
[a] Values for k_1 and K_c are apparent except as indicated: [b] $2{\cdot}57 \times 10^{-2}$; [c] $1{\cdot}58 \times 10^{-2}$; [d] $4{\cdot}10 \times 10^4$; [e] $1{\cdot}6 \times 10^4$.

The interaction of hydroxyproline (II) with the quinone is similar to that of proline but somewhat weaker as shown by the lower equilibrium constant and bathochromic absorption. This is to be expected as the hydroxy-group increases the electron positivity of the molecule. Additionally, the hydroxy-group confers greater polarity on the molecule so that the ground state of the complex should be more stabilised relative to the excited state giving rise to a blue shift of the charge transfer band.

The dipeptide, prolylglycine (III) forms a much weaker complex than proline as judged from its back rate constant and apparent equilibrium constant. As the

[11] L. Galzigna, *Nature*, 1970, **225**, 1058.

pK's of dipeptides are invariably lower than those of the *N*-terminal acid,[8] this is true for the real rate constants. In this case the weakening of the complex is probably a steric effect due to the hindrance of the *C*-terminal group,



(I) (II) (III) (IV) (V)

an effect which has also been observed for the complexes of amino-acids and dipeptides with hydroxocobalamine.[12]

Azetidine-2-carboxylic acid (IV) forms a weaker complex than proline with *p*-benzoquinone and is also relatively unstable although the charge-transfer band shows no shifts.

Thiazolidine-4-carboxylic acid (V) also forms somewhat weak unstable complexes with *p*-benzoquinone. The faster breakdown is probably mainly influenced by the sulphur atom as there is no concomitant increase in hydroquinone production as would be expected if this complex broke down in the same manner as the other complexes.

It is interesting that the stronger the complex the less likely it is to cause the reduction of the quinone to hydroquinone. As the strength of the complex is determined primarily by the charge donor, *i.e.* by reducing ability of the donor one might have thought *a priori* that the stronger donors would cause a speedier reduction of the quinone. However, as demonstrated, this is not the case but charge-transfer complexing, which is a one-electron reduction process, protects the quinone against reduction to the hydroquinone, which is a two-electron process.

Spectroscopic and thermodynamic evidence has shown that the interaction between the imino-acids and *p*-benzoquinone is due to the formation of charge-transfer complexes with the imino-acid as donor and the quinone as acceptor. This involves the re-appraisal of previous work and suggests that other systems involving the interaction of benzoquinones with amino- and imino-substituted molecules should be re-examined.

[12] J. G. Heathcote, G. H. Moxon, and M. A. Slifkin, *Spectrochim. Acta*, 1971, **27A**, 1391.

APPENDIX

For the reaction (4), where $k_1/k_2 = K_c$, $k_1([A] - [X_0]) = k_2([B] - [X_0])$ at equilibrium, and in general,

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \qquad (4)$$

equation (5) holds, where $[X_0]$ is the equilibrium value

$$\frac{d[X]}{dt} = k_1([A] - [X])([B] - [X])(1 - [X]/[X_0]) \qquad (5)$$

of [X]. For our present case where $[A] \gg [X]$ this reduces to equation (6). At $t = 0$, $d[X]/dt = k_1[A][B]$.

$$\frac{d[X]}{dt} = k_1[A]([B] - [X])(1 - [X]/[X_0]) \qquad (6)$$

When only the optical density of [X] is known, but not the true concentration, [X] is replaced by the optical density/extinction coefficient. Equation (6) is solved by simulating curves for different values of k_1 and k_2 on a computer and comparing them with the experimental curves. By obtaining curves for different concentrations, full solutions may be obtained. An independent check can be made by evaluating $k_1/k_2 = K_c$ under equilibrium conditions using the Benesi–Hildebrand equation.[5] Very good agreement was found between the equilibrium constants evaluated by the two different methods. The very good agreement between experimental and simulated curves confirms the postulated reaction scheme.

In the present case where the reactive component is the dissociated imino-acid A and not the zwitterion AH^+ then a correction must be made. A consideration of the above at $t = 0$ shows that the assumed concentration must be replaced by the real concentration of the active form. As this concentration is constant throughout the whole interaction, the scheme shown in (6) is still applicable. Thus the apparent forward reaction rate k_1 and equilibrium constant K_c must be multiplied by the ratio of the assumed to the true concentration. This is obtained from the well-known equation $pK' - pH = \log [AH^+]/[A]$. k_2 Is independent of any values assumed for A.

The measured energies and entropies are the algebraic sums of the changes involved in the dissociation of AH^+ to A and the complexing of A with B. Thus the true values of the interaction of A with B are obtained by subtracting the values of the dissociation of AH^+ from the measured values.

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