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Nonpolar Group Participation in the Denaturation of Proteins by Urea and Guanidinium Salts. Model Compound Studies[1]

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We have determined from solubility measurements the free energies of transfer of eight pure hydrocarbons from water to 7 *M* urea, and to 4.9 *M* guanidinium chloride (GuCl) over a temperature range from 2 to 50°. Six of these eight compounds represent the side chains of common amino acids. The transfer process is spontaneous owing to a positive entropy change which is large enough to override an opposing enthalpy change. For transfer to both denaturing solvents the transfer free energy becomes more negative both with increasing temperature and with increasing molecular weight of the substance transferred. Numerical values of transfer free energies (water to 7 *M* urea at 25°) range from +70 cal. for methane to −770 cal. for skatole; the range is slightly greater for 4.9 *M* GuCl. These values can be applied to the denaturation of proteins on the assumption of a plausible but as yet unproven additivity relationship. A comparison of the present results with similar studies on simple peptides shows that urea and GuCl denaturation of proteins results roughly as much from a more favorable solvation of nonpolar side chains (breaking hydrophobic bonds) as from interaction between peptide bonds and denaturing solvent.

Introduction

We are here concerned with the stabilization energy in proteins arising from so-called "hydrophobic bonds." These "bonds" are really the sum of weak, noncovalent interactions in an aqueous system containing nonpolar substances, which stabilize aggregates of the nonpolar moieties. The energetics and mechanism of hydrophobic bonding have recently been discussed by Kauzmann,[4] drawing strongly on arguments summarized by Frank and Evans.[5] Tanford[6] has more recently given a useful summary of the problem of accounting for the several sorts of stabilizing and destabilizing forces determining the structures of proteins and has made tentative estimates of the free energy of transfer of nonpolar side chains from nonpolar to aqueous environments.

In a preliminary report[7] we pointed out that our solubility measurements on skatole in aqueous urea and GuCl lead to the conclusion that these solvents break hydrophobic bonds. Almost simultaneously, Whitney and Tanford[8] reached a similar conclusion for aqueous urea and ethanol from solubility measurements on amino acids.

The experimental object of the present work has been to measure the solubilities of hydrocarbons corresponding to the side chains of several nonpolar amino acids in water and in denaturing solvents. These results have been used to calculate free energies of transfer of these substances from water to denaturing solvent. Solubility determinations have been carried out over the temperature range from 2 to 50°, covering the range in which denaturations of this sort are usually studied. We have thus obtained transfer free energies and the component enthalpies and entropies adequate for a general characterization of the transfer process, which is used as a model for the denaturation process.

Experimental

Materials.—Toluene, prepared from the sulfonic acid, was a DPI product. Skatole was obtained by twice recrystallizing a DPI sample from 50% ethanol. Its solubility was tested and it proved to be pure by the phase rule solubility test. Gaseous hydrocarbons were supplied by the Matheson Co. Propane and isobutane were Instrument Grade, 99.5% min. purity; methane, ethane, butane, and neopentane were C.P. grade, 99.0% min. purity. Commercial urea was purified by twice recrystallizing from 65% ethanol, always keeping the temperature under 40°. Purified guanidinium chloride was prepared[9] by treating the two or three times recrystallized carbonate salt with reagent grade HCl. Sodium chloride was of reagent grade; water was redistilled (glass still).

Methods.—Solutions of urea and guanidinium chloride were routinely made up to reproducible concentrations as monitored by density measurements with a Westphal balance. Dry weight determinations showed that the nominal 7 *M* urea was actually 6.96 *M*; the nominal 5 *M* guanidinium chloride was 4.87 *M*, Denaturing solutions were stored at 3° for no more than a week after preparation.

An excess of crystalline skatole was equilibrated with solvent in the apparatus described elsewhere.[10] Temperature during equilibration was controlled ±0.05°. Equilibrations were carried out for at least 48 hr. Two phase-rule tests, with six samples in each, were run on the crystalline skatole, one at the beginning of this series of solubility determinations, the other at the end. Neither showed any significant trend of solubility with varying amount of solid phase. The standard deviation from the mean in the first case was 2.4%; in the second, 0.4%.

Solubility measurements with toluene were carried out by measuring the absorbance of toluene-saturated aqueous solutions through a 2.00-mm. path-length cell. The optical cell also served as the equilibration cell. This permitted measurement of aqueous phase optical density in the presence of an excess of organic phase—a maneuver judged necessary on the basis of earlier experience which showed a substantial rate of decline in the absorbance of one-fifth saturated aqueous toluene solutions from stoppered 10-mm. cuvettes. Equilibration in the 2-mm. cell was carried out in a brass block mount in the 10-cm. cell chamber of a Beckman DU spectrophotometer. This cell chamber was provided with "thermospacers," elements for circulation of thermostat fluid, two at each end, and wrapped on all exterior surfaces with a foamed plastic insulating material. A hole to receive a thermometer bulb in the metal block cell mount was located immediately adjacent to the cell itself; an entry port through the top of the chamber permitted the bulb of a narrow-range precision thermometer to be inserted into the block during equilibration. Equilibration was hastened by application of an oscillating pressure to a Teflon capillary tube immersed in the lower (aqueous) phase of the equilibration vessel. Equilibration times were at least 15 times the half-saturation time (20 min. at 20°) and usually overnight. Before absorbance was measured, the equilibration vessel was closed and the chamber was flushed with dry nitrogen to remove radiation absorbing toluene vapors. Absorbances were measured at 2555 Å. at a constant slit width of 0.10 mm. Change of solvent produces a negligible change in toluene absorptivity at this wave length. The solubilities of toluene were obtained only in relative terms, since the molar absorptivity was not determined. The solubilities were normalized to 1 atm. vapor pressure, using the vapor pressure of pure toluene[11] and assuming Henry's law.

There is a small solubility of water and also of urea in toluene; it is therefore an approximation to use the vapor pressure of the pure compound. We have not tried to correct for this, but believe that error thus introduced is small—probably of the order of 1–2%.

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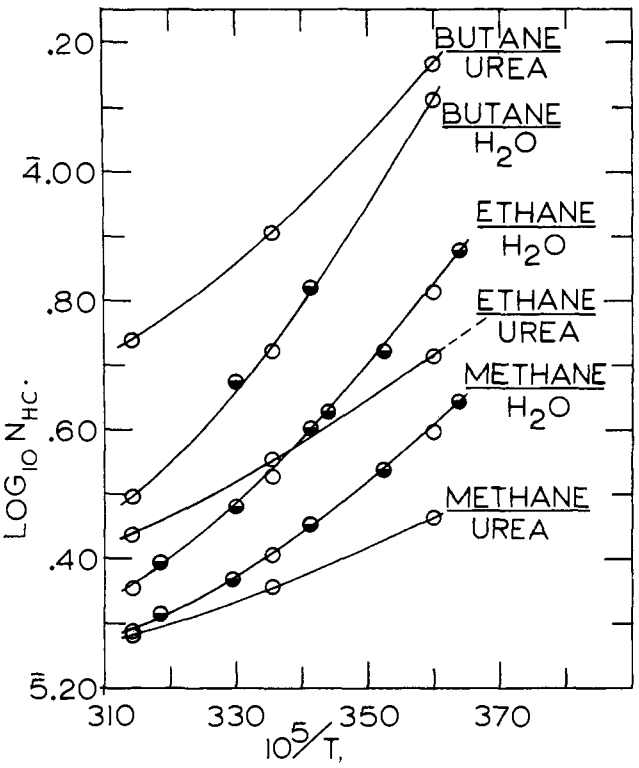

Fig. 1.—Solubility of three aliphatic hydrocarbons in water and 6.96 *M* aqueous urea; N_{HC} is the mole fraction of hydrocarbon in equilibrium with 760 mm. hydrocarbon pressure; *T* is the absolute temperature. The butane curves have been displaced upward by the addition of 0.400 to the ordinate of each point. The half-shaded points are those of Claussen and Polglase.[14]

Solubilities of the gaseous hydrocarbons were determined by means of a modified Van Slyke–Neill manometric blood gas apparatus.[12] The modifications consisted of a sample chamber with a Teflon stopcock, and agitation by a magnetic stirrer during gas extraction.

Saturation of a solvent with gas was carried out in the apparatus described elsewhere.[10] Samples were transferred to the Van Slyke extraction chamber in a rubber-tipped Mohr pipet. Calculations of solubility of the gases from the measured pressure and calibrated volume of the extracted gas were made conventionally[13] except that no corrections were attempted for the amounts of gas unextracted or redissolved. With the usual conditions of our analysis, the amount of gas unextracted can be calculated (Henry's law) to be about 1%. The percentage of gas redissolving is roughly proportional to its solubility,[13] amounting to 1.7% for cyclopropane and 0.7% for ethylene, the former being about eight times as soluble as propane, the latter three times. The re-solution error for the present studies can with confidence be estimated at well under 1%. On several trials, we have found that a second extraction yields 1–1.5% of that first obtained. This means that all of our reported results are about 1–1.5% lower than their presumed true solubilities. This is also borne out by a comparison (Fig. 1) of our determinations of the water solubility of methane, ethane, and butane with those reported by Claussen and Polglase[14] and a comparison of our nitrogen solubility determinations with those of Morrison and Billet.[15] An over-all precision of ±2% is estimated for these determinations.

Results

Representative results are shown in Fig. 1, which is a van't Hoff plot of solubility determinations for methane, ethane, and butane, in water and in 7 *M* aqueous urea. Note that the molar solubility of methane in urea is less than in water over the whole temperature range 5–45°, although an intersection at higher temperatures seems probable. For ethane, the urea and water solubility curves intersect at about 20°, while for butane the solubility in urea is greater than in water over the whole temperature range studied. In addition to showing that the effect of urea on the water solubility of alkanes depends qualitatively on the hydrocarbon molecular weight, Fig. 1 shows that the slopes of the van't Hoff plots also increase with the increasing hydrocarbon size, both for water and for aqueous urea solvent. This confirms and extends the finding of Claussen and Polglase[14] that the partial molal enthalpy change for dissolving vapor phase alkane in water increases with increasing alkane size. Note also that the slopes of the urea curves are uniformly less than those of the water curves. This is also reflected in Table II, which shows that the enthalpy of transfer from water to denaturing solvent is always positive. Finally we comment on the obvious curvature of the van't Hoff plots of Fig. 1. As was earlier pointed out for hydrocarbon in water solutions,[14,16] this means that the enthalpy of solution is somewhat temperature dependent. The curvature is seen to be less for aqueous urea than for water. The solubility results of Claussen and Polglase[14] are included in Fig. 1 for comparison: they are seen to be 1–2% higher than ours, presumably for the reasons discussed in the Experimental section. Our results with 4.9 *M* GuCl (not shown in Fig. 1) qualitatively parallel those with 7 *M* urea.

Our determination of the solubility of toluene in water as a function of temperature is very similar to that of Bohon and Claussen,[16] but a quantitative comparison cannot be made without a molar absorptivity value for toluene in water.

The detailed results of our solubility measurements are shown in Table I, which also records the numerical values of the free energy of isothermal transfer of one mole of hydrocarbon from water to denaturing solvent, ΔF°_{tr}. The standard state is here chosen as unit mole fraction of hydrocarbon behaving as at infinite dilution in water. As a consequence of this choice of standard state, the free energies and entropies obtained are identical with the "unitary" functions.[4,17] Choice of a molar or molal concentration scale has the effect of changing the location of the zero free energy coordinates of Fig. 2 and 3 without essentially altering the relationships of the several curves. We assume that the usual activity coefficient term in the free energy expression can validly be neglected since the mole fraction of dissolved hydrocarbon is in the range of 10^{-5} to 10^{-4} in the solutions here studied.

Figures 2 and 3 show the variation of ΔF°_{tr} with temperature for the eight hydrocarbons studied. First we call attention to the strong qualitative similarity between 7 *M* urea and 4.9 *M* GuCl, as shown in a comparison of Fig. 2 and 3. In a first approximation, aliphatic and aromatic compounds behave as a single family,[18] Fig. 2 and 3 showing similar curves for different hydrocarbons, the curves being displaced negatively along the ordinate in order of increasing molecular weight. The least-squares linear equations of Table II show the correlation of ΔF°_{tr} with molecular weight for all eight hydrocarbons (HC) studied. The standard deviations are less than 5% both for the $HC_{H_2O} \rightarrow HC_{urea}$ and for the $HC_{H_2O} \rightarrow HC_{GuCl}$ transfer processes. This is a surprisingly good correlation in view of the inclusion of both aliphatic and aromatic compounds.

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(18) The fact that the behavior of skatole is not exceptional in this group of compounds appears to justify its designation, for present purposes, as a "hydrocarbon."

TABLE I

SOLUBILITY OF HYDROCARBONS IN WATER AND DENATURING SOLVENTS, AND TRANSFER FREE ENERGIES

Substance	Temp., °C.	Solvent	Solubility, $M \times 10^3$[a]	$\Delta F°_{tr}$[b]	Substance	Temp., °C.	Solvent	Solubility, $M \times 10^3$[a]	$\Delta F°_{tr}$[b]
Methane	5	H_2O	2.19		Neopentane	15	H_2O	.95	
	25	H_2O	1.41			35	H_2O	.42	
	45	H_2O	1.07			45	H_2O	.34	
	5	7 *M* urea	1.31	167		15	7 *M* urea	1.03	−148
	25	7 *M* urea	1.02	69		35	7 *M* urea	0.63	−375
	45	7 *M* urea	0.86	9		45	7 *M* urea	0.54	−423
	5	4.9 *M* GuCl	1.15	197		15	4.9 *M* GuCl	1.29	−347
	25	4.9 *M* GuCl	0.96	51		35	4.9 *M* GuCl	0.67	−452
	45	4.9 *M* GuCl	0.805	−9		45	4.9 *M* GuCl	.54	−481
Ethane	5	H_2O	3.61		Toluene	10.7	H_2O	.297	
	25	H_2O	1.86			15.2	H_2O	.274	
	45	H_2O	1.25			20.0	H_2O	.279	
	5	7 *M* urea	2.33	127		29.7	H_2O	.283	
	25	7 *M* urea	1.61	−38		39.7	H_2O	.291	
	45	7 *M* urea	1.23	−122		49.6	H_2O	.328	
	5	4.9 *M* GuCl	2.28	89		11.1	7 *M* urea	.495	−405
	25	4.9 *M* GuCl	1.54	−65		15.2	7 *M* urea	.470	−427
	45	4.9 *M* GuCl	1.22	−173		20.0	7 *M* urea	.480	−437
Propane	5	H_2O	3.14			29.7	7 *M* urea	.516	−488
	25	H_2O	1.47			39.7	7 *M* urea	.580	−558
	45	H_2O	0.95			49.6	7 *M* urea	.745	−658
	5	7 *M* urea	2.49	14		10.7	4.9 *M* GuCl	.545	−505
	25	7 *M* urea	1.50	−134		15.2	4.9 *M* GuCl	.586	−604
	45	7 *M* urea	1.12	−236		20.0	4.9 *M* GuCl	.550	−572
	5	4.9 *M* GuCl	2.53	−45		29.7	4.9 *M* GuCl	.596	−633
	25	4.9 *M* GuCl	1.53	−200		39.7	4.9 *M* GuCl	.650	−684
	45	4.9 *M* GuCl	1.10	−282		49.6	4.9 *M* GuCl	.778	−746
Butane	5	H_2O	2.85		Skatole	2	H_2O	1.57	
	25	H_2O	1.16			13	H_2O	2.14	
	45	H_2O	0.69			25	H_2O	2.24	
	5	7 *M* urea	2.63	−70		37	H_2O	4.53	
	25	7 *M* urea	1.44	−251		50	H_2O	5.75	
	45	7 *M* urea	0.98	−352		37	0.15 *M* NaCl	4.37	
	5	4.9 *M* GuCl	2.89	−173		2	7 *M* urea	4.70	−708
	25	4.9 *M* GuCl	1.54	−346		13	7 *M* urea	6.57	−756
	45	4.9 *M* GuCl	1.06	−459		25	7 *M* urea	9.71	−773
Isobutane	5	H_2O	1.99			37	7 *M* urea	14 6	−848
	25	H_2O	0.94			50	7 *M* urea	21.4	−978
	45	H_2O	0.58			2	4.9 *M* GuCl	5.93	−883
	5	7 *M* urea	1.92	−95		13	4.9 *M* GuCl	8.06	−924
	25	7 *M* urea	1.13	−231		25	4.9 *M* GuCl	11.9	−948
	45	7 *M* urea	0.80	−335		37	4.9 *M* GuCl	16.2	−967
	5	4.9 *M* GuCl	1.99	−165		50	4.9 *M* GuCl	22.2	−1065
	25	4.9 *M* GuCl	1.20	−322		2	Urea–NaCl[c]	4.45	
	45	4.9 *M* GuCl	0.82	−407		13	Urea–NaCl[c]	6.19	
						25	Urea–NaCl[c]	9.31	
						37	Urea–NaCl[c]	13.6	
						50	Urea–NaCl[c]	20.0	

[a] Solubilities (except toluene) are expressed on a molar concentration scale at 1 atm. partial pressure of hydrocarbon gas and the indicated temperature. For toluene, solubilities are expressed as absorbance at 2555 Å. in a 2-mm. radiation path. [b] This function is defined as follows: $\Delta F°_{tr} = -RT \ln C_{DEN}/C_{H_2O} + RT \ln (N_{DEN}/N_{H_2O})$, where C_{DEN} and C_{H_2O} are the molar concentrations of hydrocarbon in the denaturing solvent and water, respectively; N_{DEN} and N_{H_2O} represent the moles/l. summed over all components of solvent and solute, for denaturing solvent and water, respectively. For water at 25°, $N_{H_2O} = 55.35$; for 6.96 *M* urea, $N_{DEN} = 44.95$; for 4.87 *M* GuCl, $N_{DEN} = 41.04$. [c] An aqueous solution containing 7 *M* urea and 0.15 *M* NaCl.

The standard deviation will be larger at both extremes of the temperature range since there is no systematic trend in the temperature dependence of $\Delta F°_{tr}$ with molecular weight (see Fig. 2 and 3). The correlation between $\Delta F°_{tr}$ and molecular weight must be regarded for the present as an empirical one, which fortuitously seems to work best at ordinary temperatures. On closer inspection it is seen that the aromatic hydrocarbons show a different curvature from the aliphatics in Fig. 2 and 3. This difference does not tell us anything new—only that the mechanism of solvent interaction is different for aliphatics and aromatics, a conclusion that is already evident from a comparison of the Henry's law coefficients (water solvent) for the two classes of compounds.[14,16]

TABLE II

LEAST SQUARES EQUATIONS RELATING $\Delta F°_{tr}$ AND MOLECULAR WEIGHT

For HC, H_2O, 25° → HC, 6.96 *M* urea, 25°

(a) $(\Delta F°_{tr})_{25°} = -7.32 M_{HC} + 190$ (cal./mole), $\sigma = 17.7$

For HC, H_2O, 25° → HC, 4.87 *M* GuCl, 25°

(b) $(\Delta F°_{tr})_{25°} = -8.56 M_{HC} + 183$ (cal./mole), $\sigma = 20.4$

Table III summarizes the enthalpy and entropy relationships obtained from van't Hoff plots of $\Delta F°_{tr}/T$

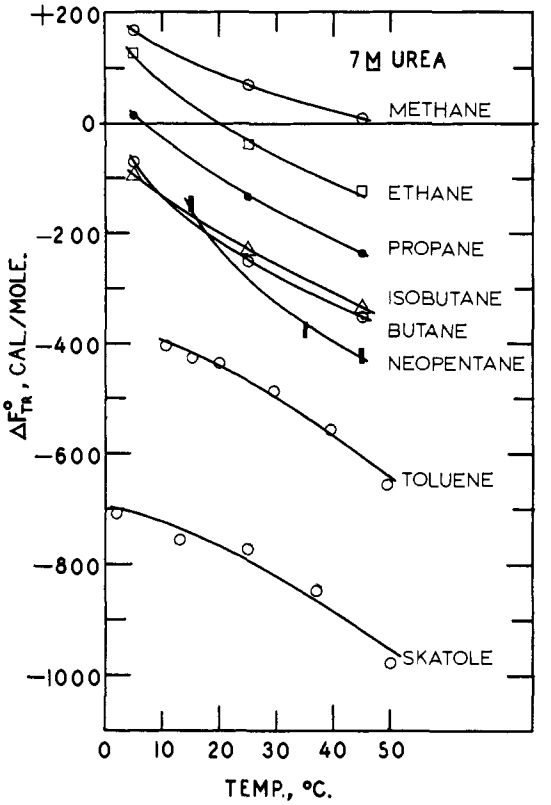

Fig. 2.—Temperature dependence of free energy of transfer of hydrocarbons from water to 6.96 *M* urea; $\Delta F°_{tr}$ is defined in the footnotes to Table I.

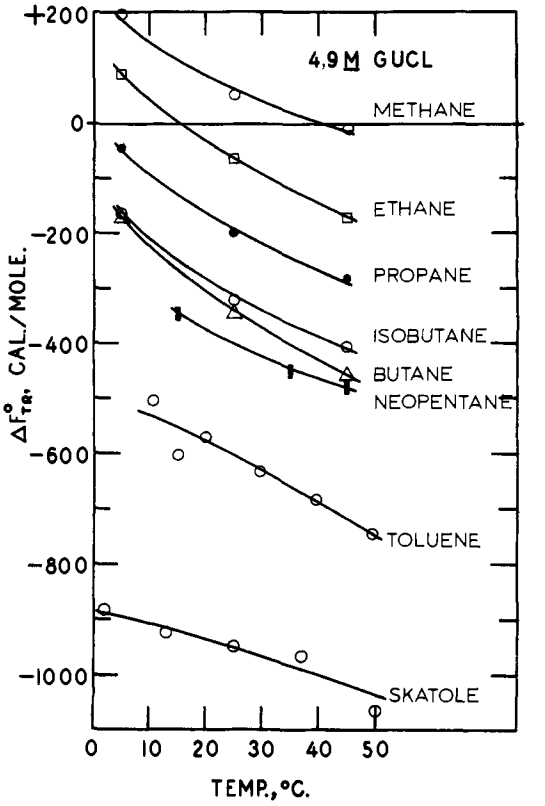

Fig. 3.—Temperature dependence of free energy of transfer of hydrocarbons from water to 4.87 *M* GuCl. See Table I footnotes for definition of $\Delta F°_{tr}$.

vs. $1/T$. These plots (not shown) are slightly curved, and the values of $\Delta H°_{tr}$ and $\Delta S°_{tr}$ are taken from the tangents of these curves at $1/298°K$. The curvature of these plots is less than that of the corresponding plots for the water solubility of the corresponding hydrocarbon (as in Fig. 1). The over-all picture of the transfer process is the striking result that transfer proceeds spontaneously from water to urea solutions in spite of an unfavorable heat, pulled as it were by the relatively large increase in entropy. The amount and quality of the data do not permit a detailed quantitative comparison of parameters for different hydrocarbons, except to mention that methane and the two aromatic compounds tend to extreme values. The process of transferring a hydrocarbon from water to aqueous GuCl is again seen to be very similar to that of transferring from water to aqueous urea.

TABLE III

SUMMARY OF THERMODYNAMIC FUNCTIONS FOR TRANSFER OF HYDROCARBON FROM WATER TO DENATURING SOLVENT (MOLE FRACTION SCALE)

	—H_2O → 7 *M* urea—			—H_2O → 4.9 *M* GuCl—		
Compound	$\Delta H°$, kcal.	$\Delta S°$, e.u.	$\Delta F°_{25°}$, kcal.	$\Delta H°$, kcal.	$\Delta S°$, e.u.	$\Delta F°_{25°}$, kcal.
Methane	1.3	4.1	+0.07	1.6	5.9	+0.05
Ethane	1.9	6.5	− .04	1.9	6.7	− .06
Propane	1.7	6.3	− .13	1.6	6.0	− .20
Butane	1.9	7.2	− .25	1.8	6.6	− .35
Isobutane	1.5	7.1	− .23	1.8	6.5	− .32
Neopentane	1.7	6.6	− .29	1.1	5.5	− .45
Toluene	1.5	6.6	− .48	0.9	4.9	− .61
Skatole	0.6	4.7	− .77	0.0	3.2	− .95

Figure 4 shows the dependence of butane solubility on denaturant concentration and presents the first evidence for dissimilarity of urea and GuCl solutions. The curvature of both the urea and GuCl lines of Fig. 4 persists when the same data are replotted on mole fraction coordinates. Up to a concentration of about 4 *M*, urea and GuCl have rather similar effects, but above this concentration there is strong divergence. The depression of butane solubility at low concentrations of GuCl is presumably a nonspecific electrolyte salting-out effect. We have observed a similar salting out with skatole at low GuCl concentrations.

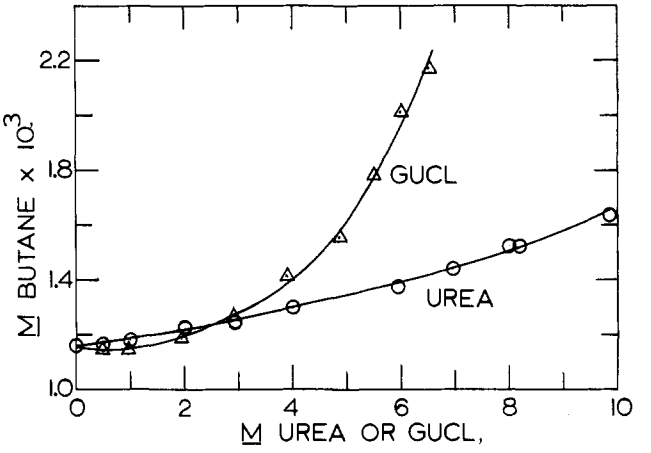

Fig. 4.—Butane solubility as a function of denaturant concentration. Both ordinate and abscissa are expressed in molar concentrations.

Two minor points deserve brief mention. The first is the small effect on $\Delta F°_{tr}$ of structural isomerism as seen in a comparison of butane and isobutane. Although there is a 20% difference in the water solubilities of these compounds, their values of $\Delta F°_{tr}$ correspond within a few per cent in both solvent-transfer systems. The second point is that, in the few experiments bearing on the question, urea and low concentrations of electrolyte (NaCl) appear to exert their effects on skatole solubility

in an approximately additive way. This is seen in a comparison of the skatole solubility (Table I) for the two solvent pairs: (a) 7 M urea, 0.15 M NaCl with (b) 7 M urea, and the solubility decrease found comparing (c) 0.15 M NaCl with (d) water. A ratio of 0.94 is obtained for (a)/(b) (mean value from five temperatures) and a ratio of 0.96 for (c)/(d) (data from 37° only).

Discussion

The stability of a protein to reversible isothermal denaturation by urea or GuCl can be considered to be determined by the ratio of the stability constants, K_1 and K_2, for the unfolding process in water (eq. 1) and in denaturing solvent (eq. 3)

$$\mathrm{Pr_{nat.}},\ \mathrm{H_2O} \rightleftharpoons \mathrm{Pr_{unf.}},\ \mathrm{H_2O} \qquad (1)$$

where

$$K_1 = [\mathrm{Pr_{nat.}},\ \mathrm{H_2O}]/[\mathrm{Pr_{unf.}},\ \mathrm{H_2O}] \qquad (2)$$

and

$$\mathrm{Pr_{nat.}},\ \text{den. solvent} \rightleftharpoons \mathrm{Pr_{unf.}},\ \text{den. solvent} \qquad (3)$$

$$K_2 = [\mathrm{Pr_{nat.}},\ \text{den. solv.}]/[\mathrm{Pr_{unf.}},\ \text{den. solv.}] \qquad (4)$$

The brackets indicate activities of the enclosed species. We further hypothesize that the free energy change corresponding to such a denaturation process can be represented as the sum of a number of terms accounting for contributions to K_2/K_1 from qualitatively different sources[19]

$$\Delta F_{\mathrm{denat.}} = -RT \ln \left(\frac{K_2}{K_1}\right)_{\mathrm{conf}} - RT \ln \left(\frac{K_2}{K_1}\right)_{\mathrm{el}} - RT \ln \left(\frac{K_2}{K_1}\right)_{\mathrm{HB}} - RT \ln \left(\frac{K_2}{K_1}\right)_{\mathrm{NP}} - \ldots \qquad (5)$$

The subscripts on the several terms indicate contributions from conformation changes, from electrostatic work done in the transition from native to unfolded protein, from changes in hydrogen bonding, and from changes in the contributions of the nonpolar groups to the free energy. Our present interest is to evaluate $(K_2/K_1)_{\mathrm{NP}}$, which we,do on the following assumptions: (a) essentially all of the nonpolar side chains are in the interior of a native protein whether the solvent is water or a denaturing solvent; (b) there is essentially complete exposure of nonpolar groups to solvent in both species: $\mathrm{Pr_{unf.}}$, unf. solvent and $\mathrm{Pr_{unf.}}$, $\mathrm{H_2O}$. As a consequence of assumption (a) it is possible to say that the nonpolar contributions to the free energy of the native protein are independent of the solvent, and therefore

$$(K_2/K_1)_{\mathrm{NP}} = [\mathrm{Pr_{unf.}},\ \text{unf. solvent}]/[\mathrm{Pr_{unf.}},\ \mathrm{H_2O}] \qquad (6)$$

Further, as a consequence of (b)

$$(\Delta F_{\mathrm{den.}})_{\mathrm{NP}} = -RT \ln \left(\frac{K_2}{K_1}\right)_{\mathrm{NP}} = \sum_{\mathrm{NP}} -RT \ln \left(\frac{N_{\mathrm{NP,\ unf.\ solv.}}}{N_{\mathrm{NP,\ H_2O}}}\right) \qquad (7)$$

where the summation is over every nonpolar side chain in the protein, and $(N_{\mathrm{NP,\ unf.\ solv.}}/N_{\mathrm{NP,\ H_2O}})$ is the mole fraction solubility ratio of a particular nonpolar compound. Combining this with the definition of our experimentally evaluated free energy of transfer (eq. 8)

$$\Delta F^\circ_{\mathrm{tr}} \equiv -RT \ln \left(\frac{N_{\mathrm{NP,\ unf.\ solv.}}}{N_{\mathrm{NP,\ H_2O}}}\right) \qquad (8)$$

we obtain

$$(\Delta F_{\mathrm{den}})_{\mathrm{NP}} = \sum_{\mathrm{NP}} \Delta F^\circ_{\mathrm{tr}} \qquad (9)$$

With a complete tabulation of $\Delta F^\circ_{\mathrm{tr}}$ for all amino acid side chains we could, by eq. 9, evaluate $(\Delta F_{\mathrm{den}})_{\mathrm{NP}}$ for the urea or GuCl denaturation of any protein of known composition and molecular weight. With the results of this paper and those of Nozaki and Tanford,[22] obtained from amino acid solubility studies, such a tabulation is well underway.

Other Estimates of $\Delta F^\circ_{\mathrm{tr}}$.—Whitney and Tanford[8] estimated $(\Delta F^\circ_{\mathrm{tr}})_{25^\circ}$ for amino acid sidechains from measurements of amino acid solubilities in water and aqueous urea solutions. These studies have been refined and extended by Nozaki and Tanford.[22] They used the assumption that

$$\Sigma \Delta f^\circ_{\mathrm{tr}} = \Delta F^\circ_{\mathrm{tr}} \qquad (10)$$

i.e., the sum of the free energies of transfer of the parts of an amino acid equals the free energy of transfer of the whole. By subtracting $\Delta F^\circ_{\mathrm{tr}}$ for glycine from $\Delta F^\circ_{\mathrm{tr}}$ for other amino acids, they obtained estimates of $\Delta f^\circ_{\mathrm{tr}}$ for these amino acid side chains. We will call this an "amino acid" measure of the free energy of transfer of amino acid side chains, in contrast with our "hydrocarbon" measure. In Table IV we have compared our results with those of Tanford and Nozaki in the four cases where this is possible. The agreement is seen to be good, with the results of Tanford and Nozaki averaging about 50 cal. more negative than ours. It is probable that this small disagreement between the two kinds of estimations is significant, since the trend of the differences is uniform. In many studies one would not be bothered by an average disagreement of 50 cal., but where this averages 10% of the total values, it is a matter for some concern.

TABLE IV

COMPARISON OF "HYDROCARBON" AND "AMINO ACID" MEASURES OF $(\Delta F^\circ_{\mathrm{tr}})_{25^\circ}$ FROM $\mathrm{H_2O}$ TO 6.96 M UREA

Nozaki and Tanford[22]	$(\Delta F^\circ_{\mathrm{tr}})_{25^\circ}$ a.a. value	This work	$(\Delta F^\circ_{\mathrm{tr}})_{25^\circ}$ HC value	HC − a.a. value
Side chain of Ala	+11[a]	Methane	+69	−58
Side chain of Leu	−258[a]	Isobutane	−231	−27
Side chain of Phe	−533[a]	Toluene	−481[b]	−52
Side chain of Try	−821[a]	Skatole	−773[b]	−48

[a] These values were obtained by a linear interpolation between the values at 6 and 8 M found by Tanford and Nozaki. [b] These values were obtained from a graphical interpolation in linear plots of log N_{HC} *vs.* $1/T$.

We shall raise two questions in this connection. First, does the solubility of glycine in strong urea provide precisely the right base line? Second, does the principle of additivity really hold for the amino acids under study? Nozaki and Tanford assumed that the self-interaction of a particular amino acid is the same in aqueous urea as in water. They note that their treatment of the activities of the amino acids in water and urea is oversimplified but (properly for their purposes) consider it an adequate first approximation. It can be argued, however, that the actual error from their treatment would be largest with the most soluble amino acid, glycine (saturated aqueous glycine is 2.9 M at 25°) and it was glycine that was used to set the "base line" in their experiments. If we should, for example, consider Nozaki and Tanford's $\Delta F^\circ_{\mathrm{tr}}$ values for glycine at urea concentrations > 4 M to be less reliable than their values found for the more dilute urea solutions, and carry out a linear extrapolation of these latter values to 6.96 M urea, this would make the glycine base line 36 cal. more positive, reducing the average differences of Table IV from −46 to −10 cal. But we do not really know that it is correct to do this, any more than we really know that our results are more nearly correct than those of Nozaki and Tanford.

(19) Conceptually similar formulations have recently been employed by Tanford,[6] by Scheraga,[20] and by Brandts and Lumry.[21]

(20) H. A. Scheraga in "Polyamino Acids, Peptides, and Proteins," M. A. Stahmann, Ed., Univ. of Wisconsin Press, Madison, Wis., 1962, p. 241.

(21) J. Brandts and R. Lumry, *J. Phys. Chem.*, **67**, 1484 (1963).

(22) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, in press.

Another way of interpreting the differences between the two sets of results is to question the additivity assumption of eq. 10. Many of the properties of proteins can be expressed to a good first approximation as the simple sum of the properties of the component amino acid residues, but good quantitative agreement with experiment usually requires a more elaborate combinatorial equation. The properties, specific volume, electronic absorbancy, and H^+ equilibrium represent cases in point.

There is, in fact, a conceptual difficulty with the idea that the solvent interactions of amino acid side chains and the dipolar ion (glycine part of the molecule) should be independent and additive. There are strong reasons to believe[4,5] that the structure of water is different in the near neighborhood of a nonpolar solute from that in pure water. Moreover, water takes still another structural mode in the neighborhood of ions (including dipolar ions). If we now make an imaginary attachment of the nonpolar group to the dipolar ion, we see that highly dissimilar solvent domains are brought into contact and, indeed, tend to overlap to some extent. This could lead to failure of a two-term additivity expression. On the other hand, if this juxtaposition of two dissimilar solvent domains makes little difference to the net energy of the nonpolar amino acid as compared with its "separated parts," a two-term additivity expression will adequately express the experimentally observed transfer energies. It appears that, in our conceptual model, the overlap of solvent domains should be of greatest consequence when the nonpolar side chain is either small, as in alanine (producing a greater effect relative to the whole molecule), or when it is branched on the β-carbon atom (producing a greater overlap volume) as in valine and isoleucine. These conjectures are clearly amenable to experimental test, and such experiments have been begun in our laboratory.

In addition to the above argument about overlap of solvent domains, we could perhaps expect some small difference in behavior of a nonpolar side chain and the corresponding hydrocarbon. These two species differ, of course, in that the side chain has one hydrogen less than the hydrocarbon. The critical region of an amino acid (or amino acid residue in a protein) where the side chain is linked to the α-carbon atom is still under scrutiny, but now for the purpose of indicating the nonidentity of the model systems employed in the "amino acid" approach and the "hydrocarbon" approach. If this difference of an H atom has any practical significance, the "amino acid" model system will be somewhat better than ours in this one respect.

It is of course possible that neither our present studies nor those of the sort employed by Nozaki and Tanford are wholly adequate model systems for evaluating the contributions of nonpolar side chains *attached to a peptide main chain*. Solubility determinations for various peptides in water and denaturing solvents should help provide an answer to this question.

Mechanism of Action of Urea and GuCl.—We do not need to know anything about the mechanism of hydrocarbon solubilization to make use of the values of ΔF°_{tr} here obtained. We find the question interesting nonetheless and will briefly comment on it.

First we should like to know whether urea and GuCl operate by the same mechanism. There is substantial evidence[23,24] that in protein denaturation they do. Moreover, as is clear from an inspection of Fig. 2 and 3, the two reagents have very similar effects on hydrocarbon solubilities. It is only in the concentration-dependent functions (Fig. 4) that differences appear. Since the GuCl curve of Fig. 4 includes an indeterminate "salting-out" component and the urea curve does not, we cannot interpret Fig. 4 as compelling evidence for different mechanisms in the actions of urea and GuCl.

We suggest three mechanisms for the increased solubility of hydrocarbons in aqueous urea: (a) dissolved hydrocarbon is solvated solely by urea molecules; (b) urea alters the structure of water so as to facilitate solvation of the hydrocarbon by water molecules; (c) hydrocarbon molecules are solvated both by urea and by water. "Solvation" in this context is used only to designate the near neighbors of the dissolved hydrocarbon and carries no further connotation.

The fact of a linear dependence between ΔF°_{tr} and M_{HC} (Table II) implies that for the larger hydrocarbons, a urea–hydrocarbon complex (mechanism a) should contain more than one urea molecule per hydrocarbon molecule. This in turn would require a stronger dependence of solubility on urea concentration than that observed in Fig. 4. This argument is sufficient to dismiss mechanism (a). The apparently higher-order dependence on GuCl above 4 M (Fig. 4) cannot be interpreted as favoring mechanism (a) since the GuCl curve contains a salting-out component of unknown character and magnitude.

At present we do not see any way of discriminating between a facilitating mechanism (b) and a cooperative mechanism (c). This question will be deferred to a later publication.

Comparison of Peptide Backbone and Nonpolar Side Chains in Urea Denaturation.—Data for ΔF°_{tr} of the glycyl *residue* have been obtained by Nozaki and Tanford[22] and by Robinson and Jencks.[25] These results are, in both cases, only for the process at 25°. Although there is considerable variation in the values, depending on the particular compound chosen for the evaluation, it appears fair to choose an average value of -127 cal. ($7/8 \times -145$ cal. (8 M urea)[22]) for transfer of a glycyl residue in a peptide from water to 7 M urea. The values for the comparable process for nonpolar side chains range (Table III) from $+80$ cal. to -770 cal. The sum of the contributions of the nonpolar side chains will obviously depend in detail on the composition of the protein in question.

To get a rough idea of the relative contribution of the nonpolar side chains, we have proceeded as follows. Validity is assumed for eq. 5, 7, 9, and 10. These nine amino acid side chains are considered to be transferred from water to 7 M urea at 25°: Ala, Val, Pro, Leu, Ileu, Met, Phe, Tyr, and Try.[26] The mean side-chain weight for this group is 68. Assuming that all these amino acids are present to the same extent, and that they all contribute according to eq. a of Table II, we obtain -310 cal. as the mean nonpolar chain contribution. Using Tristram's compositional data[27] for an average of 40 proteins, we obtain an average value of 40% nonpolar residues. This yields an average of -124 cal./residue averaged over all amino acid residues of an average protein. Within the limits of the approximations just made, we can expect about equal contributions to the free energy of urea denaturation from the nonpolar side chains and from the peptide main chain. Note that this comparison can only be made at 25°, and that the values of ΔF°_{tr} for a glycyl residue are less certain than those for the nonpolar side chains.

(23) H. Neurath, J. P. Greenstein, F. W. Putnam, and J. O. Erickson, *Chem. Rev.*, **34**, 157 (1944).

(24) J. Schellman, R. B. Simpson, and W. Kauzmann, *J. Am. Chem. Soc.*, **75**, 5152 (1953).

(25) D. R. Robinson and W. P. Jencks, *J. Biol. Chem.*, **238**, PC 1558 (1963).

(26) The data of Nozaki and Tanford[22] appear to justify the inclusion of the side chains of Met and Tyr in the nonpolar group for present purposes.

(27) G. R. Tristram, in "The Proteins," H. Neurath and K. Bailey, Ed., Academic Press, Inc., New York, N. Y., 1953, Vol. 1, Part A, p. 181.

Micelle Studies.—In two recent studies[28,29] detergent micelles have been used to evaluate the effect of urea on hydrophobic bonds. The main finding of these studies was that urea raises the critical micelle concentration (c.m.c.). Although no quantitative estimates were made, both groups of workers implied that urea has rather little ability to break hydrophobic bonds. From measurements of c.m.c. dependence on urea concentration at two temperatures, Mukerjee and Ray also concluded that there is no temperature dependence in the hydrophobic bond-breaking tendency of urea. Our present results are at variance with these conclusions. Although micelle formation most certainly does involve hydrophobic bonds, it appears to us that it does not provide a suitable model system for this type of study. The reason for our objection is that the micelle is not the same as a solid crystalline phase in the sense of having constant composition, constant structure, and especially constant free energy. Thus, when we write an equation for micelle formation: n detergent $\rightleftharpoons$ $(\text{Det})_n + \Delta F$, and consider how change of solvent composition affects the equilibrium, we must assume that a change in the equilibrium is due only to change in the chemical potential of the "free-swimming" detergent molecules (those on the left side of the equation) and not of the micelles, if we are to calculate the change in ΔF from the change in c.m.c. Since it is generally accepted that the capacity of micelles to vary in structure is an important feature of detergency, this assumption is dubious. In other words, we question that the chemical potential of $(\text{Det})_n$ does indeed remain constant with a change in solvent composition. Even if the foregoing objection is met, we submit that a change in the chemical potential of the free-swimming molecules will have contributions from both the polar and nonpolar parts of the molecules. Arriving at this point, one must evaluate the relative contributions from both parts of the molecule to get an estimate of the interaction between the nonpolar part of the molecule and the solvent. Since the micelle studies cited did not account for these considerations, we believe that their interpretation is subject to much uncertainty.

(28) W. Bruning and A. Holtzer, *J. Am. Chem. Soc.*, **83**, 4865 (1961).
(29) P. Mukerjee and A. Ray, *J. Phys. Chem.*, **67**, 190 (1963).

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COMMUNICATIONS TO THE EDITOR

A New Univalent Cobalt Complex

Sir:

Previously cobalt(I) ion was found by polarographic means to exist in solution as a stable ion.[1]

We have now succeeded in isolating *in vacuo* the phenanthroline cobalt(I) perchlorate from the solution as dark brown crystals. It was found to be the hexacoordinated complex, $[\text{Co}^{\text{I}}(\text{phen})_3]\text{ClO}_4$.[2]

A reaction apparatus fitted with fritted joints to allow a series of procedures such as mixing, filtering, washing, and sealing *in vacuo* was attached to the usual type vacuum line with an oil diffusion pump which could obtain a pressure of 10^{-7} mm. Sodium borohydride was used as the reducing agent. All procedures were carried out *in vacuo*.

Tris(phenanthroline)cobalt(II) perchlorate (1 g.) was dissolved in about 200 ml. of 10% ethanol–water mixture and it was frozen with liquid nitrogen. The space above the frozen solid was evacuated. After the stopcock was shut, the frozen solid was melted with an electric air drier. The air which was dissolved in the solution bubbled vigorously. The solution was again frozen and the space above the solid was evacuated. This procedure was repeated three times. Under these conditions all oxygen was removed from the reaction mixture.

Similarly the aqueous solution of sodium borohydride (0.5 g.) and the wash solution, containing no dissolved oxygen, were prepared in the vessels connected to the reaction flask. The luteo-salt and the borohydride ion were allowed to react gently at −5°. The color of the solution changed from yellow to brown and a brownish black powder separated from the solution. This was filtered and washed *in vacuo*. The product on the glass filter (G3) was recrystallized by dissolving in the deaerated 80% ethanol–ether mixture. The solvent was evaporated by suction and lusterous dark brown crystals deposited on the wall of the vacuum tube.

Anal. Calcd. for $[\text{Co}^{\text{I}}(\text{phen})_3]\text{ClO}_4$: Co, 8.43; C, 61.86; N, 12.02; H, 3.46. Found: Co, 8.32; C, 61.97; N, 11.92; H, 3.21.

The complex is soluble in ethanol and ether and insoluble in water. The aqueous solution of the complex was oxidized with 10% hydrogen peroxide and polarographic studies indicated it to be the $[\text{Co}^{\text{I}}(\text{phen})_3]^{+3}$ ion.

The four-coordinated complex, $[\text{Co}^{\text{I}}(\text{phen})_2]\text{ClO}_4$, could not be obtained under any reaction conditions reported for the dipyridyl cobalt(I) complex.[3,4]

(1) N. Maki, T. Hirano, and S. Musha, *Bull. Chem. Soc. Japan*, **36**, 756 (1963).
(2) phen = 1,10-phenanthroline. This result was presented at the 13th Symposium on Coordination Compounds, Nagoya University, Nagoya, Japan, Oct. 15, 1963.
(3) A. A. Vlček, *Nature*, **180**, 573 (1957); *Z. physik. Chem. Sonderheft* (Internationales Polarographisches Kolloquium, Dresden), 143 (1958).
(4) G. M. Waind and B. Martin, *J. Inorg. Nucl. Chem.*, **8**, 551 (1958).
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RECEIVED NOVEMBER 30, 1963

The Reaction of Trialkyl Phosphites with Aliphatic Aldehydes. P^{31} and H^1 Nuclear Magnetic Resonance Spectra of Tetraoxyalkyl Phosphoranes[1,2]

Sir:

We wish to report the isolation and characterization of a tetraoxyalkyl phosphorane(I) from the reaction of 3 moles of anhydrous propionaldehyde with 1 mole of trimethyl phosphite at 20°. The 2:1 adduct

(1) (a) F. Ramirez, A. V. Patwardhan, N. B. Desai, N. Ramanathan, and C. V. Greco, *J. Am. Chem. Soc.*, **85**, 3056 (1963); (b) F. Ramirez, N. Ramanathan, and N. B. Desai, *ibid.*, **85**, 3465 (1963); (c) F. Ramirez and N. B. Desai, *ibid.*, **85**, 3252 (1963); **82**, 2652 (1960).
(2) Acknowledgment is made to the Cancer Institute of the National Institutes of Health (CY-04769) and to the National Science Foundation (G19509) for support of this research.