bonds is negligible, were not successful because of a large amount of scatter in the experimental results. If such measurements could be made, they might be a better confirmation of the value of $\Delta H_{res.}^0$ than measurements in the neutral *p*H region. The effect of decomposition of urea should also be minimized at low *p*H. However, the effect of charges on the protein might not be eliminated even at this high ionic strength and might be serious at low *p*H.

This study was undertaken concurrently with similar studies of insulin fibers and ribonuclease films[6] and the three should be considered as complementary. The case of tanned ribonuclease is especially interesting in view of its practically complete reversibility in diluents of high salt concentration and low *p*H. In fact, the reversibility of the transition previously noted in studies of ribonuclease solutions[7,57] was a strong factor in motivating a successful demonstration of reversibility of the transition in the cross-linked ribonuclease network. This in turn lends support to the postulate that, in the case of fibrin, the transition also may be truly thermodynamically reversible. That the transition is thermodynamically reversible has been shown to be probable, but was not proved, in this work.

The values of the significant parameters which result from the treatments are quite similar, and this encourages us to consider them, at least tentatively, to be generally applicable. The case of fibrin demonstrates their applicability to a system which was not previously subjected to the action of reagents which change the covalent structure of the molecule, as quinone or formaldehyde may.

(57) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. lab. Carlsberg Ser. chim.*, **30**, 167 (1957).

These parameters fall into two classes. Those which are included in ΔF_H^0 are attributed to the ionization and hydrogen bonding of side-chain ionizable groups. Those which are included in ΔF_b^0, however, are based on rather arbitrary assumptions about protein structure, which may or may not be valid. The fact that our selection of the parameters $\Delta H_{res.}^0$ and $\Delta S_{res.}^0$, and a reasonable length for the "average" chain, leads to reasonable values of ΔF_b^0 may be fortuitous; it would, therefore, be misleading to take this as evidence for the α-helical conformation in this system. We have shown that the α-helical model, with reasonable values of the parameters, does give agreement with the experimental results; however, other models for the backbone structure, which have the same *p*H-independent values of ΔF_b^0 will also give satisfactory agreement. This qualification does *not* pertain to the significance of the good agreement for the *pH-dependent* aspect of the transition (Fig. 3).

The secondary structure of fibrin, as evidenced by the X-ray studies of several workers (see review by Low[9]) must undergo drastic changes during the transition. This lends support to the model used here in the sense that we may be confident that we are indeed observing changes in the secondary structure of the molecule, and not merely observing disorientation of the molecular units which have aggregated to form the elastomer and then have been forced into an oriented configuration by physical treatment.

Acknowledgment.—We are indebted to Drs. P. J. Flory, L. Mandelkern, A. Nakajima, and J. F. M. Oth for helpful discussions of this problem, and to Mrs. Miriam Taylor for aid in the experimental work.

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Studies of Rack Mechanisms in Heme-proteins. I. The Magnetic Susceptibility of Cytochrome c in Relation to Hydration

BY RUFUS LUMRY, AAGE SOLBAKKEN, JOHN SULLIVAN AND LLOYD H. REYERSON

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The magnetic susceptibilities of trypsinogen, oxidized cytochrome *c* (OCy) and reduced cytochrome *c* (RCy) have been determined as a function of the amount of water bound starting with the lyophilized material. The susceptibility change of trypsinogen is entirely due to bound water which maintains the susceptibility value of liquid water. The susceptibility of the protein does not change with the addition of bound water. Dry OCy and RCy have higher susceptibilities than hydrated or dissolved forms. This change is due to unpairing of spins in RCy and the same explanation is probably correct for OCy. The changes in electronic state of the iron ions is interpreted as being due to shifts from 1 to 3 unpaired electrons in OCy and from 0 to 2 unpaired electrons in RCy and attributed to changes in the fields due to imidazole ligands as their positions are altered by rearrangements in the protein conformation. This situation is interpreted as a rack mechanism in which the protein conformation controls in part the electronic state of the heme group and several possible consequences of this mechanism are presented. Cytochromes dried by lyophilization have different susceptibilities from those dried by water removal at 27° and the difference may result from the displacement of one or more imidazolium groups by water in the iron coördination shell.

The magnetic susceptibility of cytochrome *c* was first investigated in 1940 by Theorell[1] who used impure preparations of horse-heart and cow-heart cytochrome *c*. In studies at 22° he found a wide range of paramagnetic susceptibility values for ferricytochrome *c*, OCy, on varying the hydrogen-ion concentration. In the neutral range of *p*H this compound yielded values from 2580 to 3340 $\times 10^{-6}$ c.g.s. units for χ. Van Vleck[2] and, more recently, Griffith[3] have pointed out the importance

(1) H. Theorell, *J. Am. Chem. Soc.*, **63**, 1820 (1941).

(2) J. H. Van Vleck, "The Theory of Electric and Magnetic Susceptibilities," Oxford, 1932.

(3) J. S. Griffith, *Biochem. et Biophys. Acta*, **28**, 439 (1958).

of measuring the temperature dependence of the susceptibility prior to its interpretation. Theorell interpreted his results on the form at neutral *p*H, type III, as being due to a contribution from one unpaired electron (1260×10^{-6} contribution to χ) plus a large orbital supplement which varied from 1320×10^{-6} to 2080×10^{-6} in his experiments. His data for ferrocytochrome *c*, RCy, gave a low susceptibility and were assumed to indicate a diamagnetic state. At neutral hydrogen-ion concentration the iron ion is bound to four pyrrole nitrogens and presumably to two imidazole nitrogens furnished by the globin portion of the molecule. Large changes in *p*H force displacements of at least one of the imidazole ligands by water or hydroxyl ions probably through a rupture of protein-heme bonds and certain inhibitors will also displace one and perhaps two of these ligands. Theorell's studies emphasized the drastic changes in the susceptibility of the heme group which occur on changing ligands in the fifth and sixth positions. It is to be noted that there may be a more delicate and more subtle way for varying the electronic properties at heme iron which does not involve changes in ligand. The positions of the fifth and sixth ligands in type III cytochrome *c* probably represent a compromise situation of lowest total free energy for a given set of solution conditions in which the ligands are oriented and placed in positions which are neither those to be expected in the corresponding hemochromogen nor those which allow the most stable folding of the protein. The electronic properties of the heme group and especially the complexed iron ion could thus be controlled to some small but highly important extent by the conformation of the protein which forces a change in imidazole orientation, a change in iron-nitrogen bond length or a change in angle of imidazole-iron bond to the heme plane. Furthermore, there may be a strong "trans-effect" interaction between the two imidazole groups as well as a protein-controlled inductive effect through the imidazole ring and depending on the nature of the groups near the second nitrogen atoms, *i.e.*, those not bound to iron, of the imidazole groups. On the basis of this so-called "rack" model[4] changes in the protein conformation either induced by changes in solution conditions or by genetic alterations in the nature of one or more key amino acid residues should alter the ligand-field effect of the imidazole groups on the iron ion and such alterations should appear as changes in the electronic properties of the heme group. The latter properties can be measured by spectral studies, by nuclear magnetic resonance relaxation methods when the heme is not diamagnetic, by measurement of the Cotton effect in the visible region of the heme spectrum, by determination of the oxidation-reduction potential or by studies of the magnetic susceptibility using either electron spin resonance techniques or direct determination of static susceptibilities. In subsequent papers the results of studies with the several methods will be reported. In the present publication we give the findings of static susceptibility

(4) R. Lumry and H. Eyring, *J. Phys. Chem.*, **58**, 110 (1954).

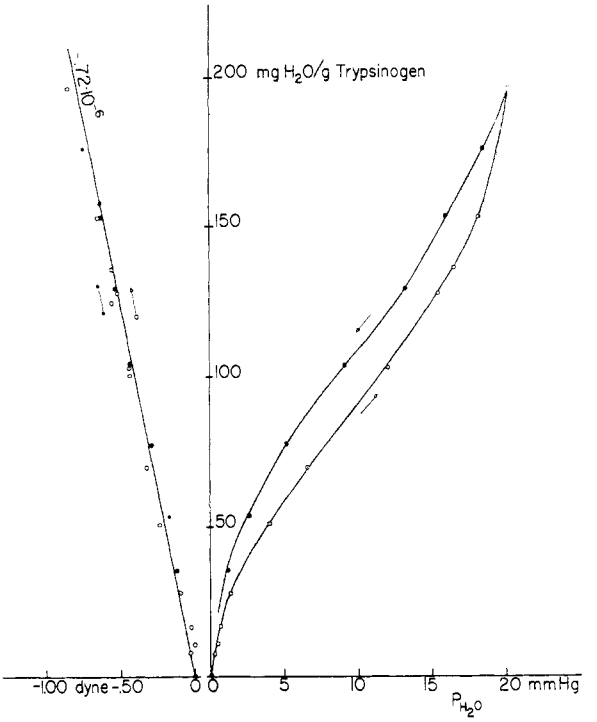

Fig. 1.—Absorption isotherms and magnetic susceptibilities of trypsinogen as a function of the amount of water bound. Susceptibilities are given in terms of the relative force on the sample due to the magnetic field. The experiment started with a lyophilized protein sample. Temperature was 27°.

experiments carried out with different amounts of water held to the protein. Cytochrome *c*, because of the fact that both fifth and sixth-position ligands are provided by the protein, is particularly useful for investigations of the rack mechanism. On removing water or carrying out other drastic changes in solution conditions other than the introduction of strongly bound inhibitors, we may expect the imidazole groups to persist as ligands. Removal of water can be expected to produce large modifications in protein conformation without perhaps forcing direct changes of ligands, and is a sufficiently drastic yet reversible process to test the rack hypothesis as applied to heme proteins in a first, gross way.

Results

Trypsinogen.—As a control, the magnetic susceptibility of pure trypsinogen was determined as a function of the amount of water bound. The absorption and desorption isotherms for this protein are shown to the right of Fig. 1 and are very similar to what is usually observed for proteins. The observed values of the relative magnetic susceptibility measured in dynes are plotted on the left-hand side of this figure and the solid line is the diamagnetic susceptibility curve for water adjusted to fit the zero-water point. Although the precision was not so high in this experiment as in the other experiments, there seems to be little doubt that the increasing diamagnetism of the sample with increased hydration is due entirely to the bound water and

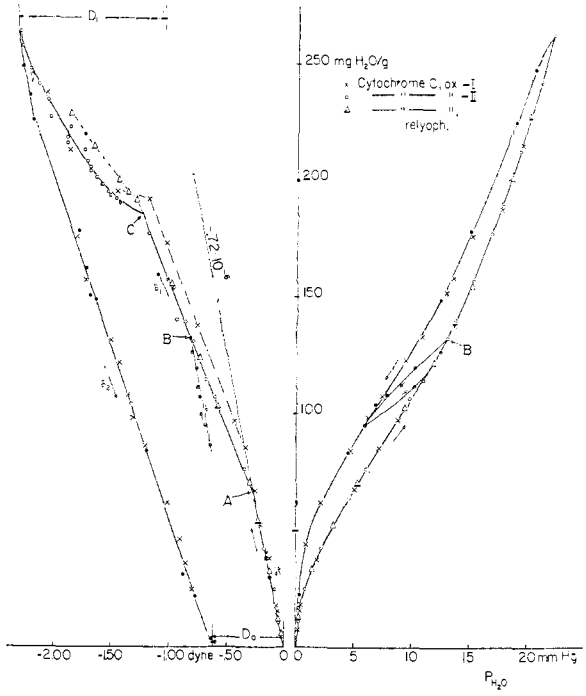

Fig. 2.—Absorption isotherms and magnetic susceptibilities of oxidized cytochrome *c* as a function of the amount of water bound. Susceptibilities are given in terms of the relative force on the sample due to the magnetic field. X-points obtained with a freshly lyophilized sample; Δ-points obtained on recycling the first sample; ●- and ○-points obtained with a second sample. 27°. See text for full description.

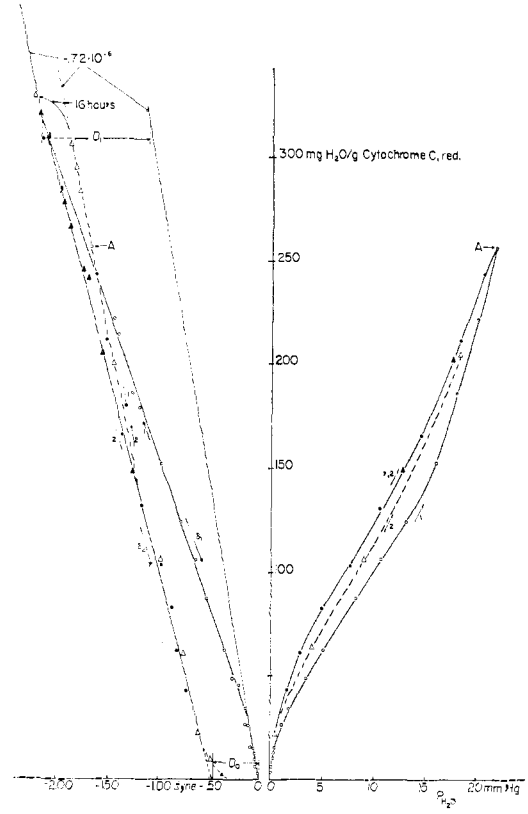

Fig. 3.—Absorption isotherms and magnetic susceptibilities of reduced cytochrome *c* as a function of the amount of water bound. Susceptibilities are given in terms of the relative force on the sample due to the magnetic field: ○-points obtained on first hydration of a freshly lyophilized sample; ●-points obtained during the first dehydration process in the balance; Δ-points obtained during the second hydration of the original sample; ▲-points obtained during the second dehydration process of the original sample. 27°.

that the susceptibility of protein-bound water is identical to that of free water. Conceivably there could have been compensating effects accidentally yielding the water line but we have no evidence that this is the case.

Oxidized Cytochrome *c*.—Samples of OCy lyophilized in a perfectly normal manner were transferred in air to the sample holder of the balance. The absorption and desorption isotherms are given in the right of Fig. 2. Although the data are too fragmentary to include, it was observed that the second absorption curve lay closer to the desorption curve which did not change during hydration cycles. The hysteresis area between the isotherms was thus reduced. This situation was also found with RCy samples as shown in Fig. 3, and the hysteresis area is considerably smaller than usually observed with proteins.[5] The lack of reproducibility of the absorption isotherm has been observed repeatedly by Reyerson and Hnojewyj with lysozyme, β-lactoglobulin and chymotrypsinogen either in comparing the first absorption curve for a new protein sample with the second such curve, or on reducing the temperature in a system which has previously passed through the hydration cycle at a higher temperature. Third and fourth absorption curves were identical with the second. In both situations this behavior can for the present be interpreted as meaning that the conformation of the protein changes during hydration. In the present example it may be concluded that the state of the protein, presumably its conformation, is different in a dry material prepared by lyophilization than it is in material dryed by evacuation at 27°. The susceptibility plotted to the left of Fig. 2 shows similar behavior. Irreversibility of this sort as measured by hydration and susceptibility was checked by re-lyophilizing OCy and RCy following the hydration cycle. The behavior of these samples in subsequent hydration cycles was very nearly identical with their initial behavior and a fresh sample of OCy behaved in a fashion identical with that of the first.

The first part of the susceptibility curves of Fig. 2 from 0 to A has the slope of normal bulk water and desorption from A to 0 followed the same line. The second segment of the absorption curve, from A to B, has a larger slope than the water line indicating, on the basis of the trypsinogen results above, that the diamagnetism of the sample is increasing by amounts greater than can be attributed to the increase in bound water. However, reversal from point B by pumping off water follows a line with the water slope. A consequence of this behavior which we shall draw tentatively

(5) W. S. Hnojewyj and L. H. Reyerson, *J. Phys. Chem.*, **63**, 1653 (1959); L. H. Reyerson and W. S. Hnojewyj, *ibid.*, **64**, 811 (1960); W. S. Hnojewyj and L. H. Reyerson, *ibid.*, **65**, 1694 (1961).

is that the true zero point is not given by 0 of this figure but rather lies at the point at which the line CBA intersects the abscissa. Part of the irreversible state of the protein produced by lyophilization appears to relax to a new form characterized by the line CBA with the smallest traces of bound water, and since the protein was briefly exposed to wet room air in transfer to the balance, we may for the time being attribute the existence of segment OA to this exposure to water. To some extent this conclusion is borne out by the fact that different treatments of the same sample give different lengths of this initial segment as shown by the X points on the dashed line. The sample with X points was exposed to air for a different length of time during transfer than it was during the second transfer (Δ points). Nevertheless, there are some non-reproducible features of the segment which indicate that the behavior of different samples is not strictly identical, even if one corrects for the different lengths of the OA segment. These are best seen at point C. The points X are from the first sample; those marked with open triangles from relyophilization of the first sample; and those indicated by dots from a new sample. The break in the curves near point C is seen to vary with the sample and the experiment. This is probably a rate phenomenon since equilibration is reached very slowly at this point. It is noteworthy, however, that the triangles and the crosses do not superimpose in the region from C to saturation, even following adjustment for the differences in the segments OA.

All samples demonstrated a break though of varying sharpness near point C. The precision of fixing the break points is not much less than their spread. Beyond the break region all samples increased in diamagnetism rapidly with increasing amounts of bound water. The dots and crosses reached the same value at water pressures within a few mm. of water saturation at this temperature. The susceptibility was still decreasing with increase in bound water at the highest water pressures which were studied, though the rate of change with increasing water was slow. It appears that the absorption curve is asymptotic to a line with the water slope at these high pressures.

As water was removed, the return susceptibility line, marked δ_2, first followed a line with the water slope and then a line with greater slope indicating a more rapid decrease in diamagnetism or increase in paramagnetism than can be accounted for on the basis of the removal of bound water. In no case was it possible to return by evacuation of the system to point 0, although some samples were pumped for as long as six days at 10^{-6} mm. At the end of this time 1.5 molecules of water per molecule of protein (mol. wt. = 12,300) relative to any initial content at point 0 remained on the protein. This tenacity for water is not observed in coenzyme-free proteins.[5]

Reduced Cytochrome *c*.—A very similar set of experiments was carried out with RCy and the results are given in Fig. 3. The absorption- and desorption-isotherm behavior has been discussed above. After an initial region of hydration indicating a susceptibility decrease due to water, the susceptibility followed line δ_1 with slope greater than the water value to point A. As indicated by the arrow, desorption from this point followed a line of water slope back to its intersection at about 140 mg./g. of water with the solid desorption line and from that point followed the desorption line, δ_2, back to about 15 mg. water/g. As with OCy, desorption beyond this point was extremely slow but it did occur until about 0.92 molecule of H_2O per molecule remained. The susceptibility at this point was less than at point 0 but its marked rise with decreasing water in this region suggests that with sufficient time the protein might have been totally dehydrated to the susceptibility value at 0.

Water was reabsorbed onto the protein with results shown as triangles. Reabsorption faithfully followed the desorption curve to the point of intersection with the line of water slope at about 140 mg. H_2O/g., at which point it deviated as shown by upward pointing arrow 2 passing first through A and then following this line with the water slope to about 300 mg./g. Up to this point no break such as that observed near C (Fig. 2) for OCy was observed but this appears to be a kinetic phenomenon due to our failure to hold the sample at constant vapor pressure for a sufficient length of time. That this is so is shown by the sharp increase in diamagnetism which occurs in the region marked 16 hr., this time being the time of equilibration for this point. This final triangle appears to lie on a line with the water slope. In a subsequent experiment carried out to verify the results obtained with this experiment the water vapor pressure was raised to its saturation value. The protein went into solution and the solution had a total susceptibility value including the water contribution lying on this line.

Dehydration in the first experiment from the last open triangle is indicated by the closed triangles and follows the desorption line δ_2 in the direction of the downward pointing arrow 2 all the way back to the lowest pressures. The results with this protein are thus very similar to those obtained with OCy with the possible exception of the omission of the sharp break found near C for the latter.

Estimation of the Susceptibilities.—It was not possible because of the geometric factors involved in positioning the sample bucket in the magnetic field to make accurate estimates of the absolute values of the susceptibility of the protein samples. We have thus assumed the average value of the susceptibility of OCy determined by Theorell in solution as a base point for OCy and a totally diamagnetic state as the base point for RCy. Using the susceptibility of bound water at 27° (-0.72×10^{-6} c.g.s. units[6]) and the differences, $\Delta\chi$, which are determinable with some precision in our experiments, it is possible to estimate the absolute susceptibility at each point. Such calculations have been made for the points 0 using D_1, for the lower end of the desorption curves using D_0 and for the points at which the lines δ_1 intercept the abscissa. These estimates and the slopes of

(6) Data from "International Critical Tables," Vol. VI, McGraw-Hill Book Co., New York, N. Y., 1929.

TABLE I

PARAMAGNETIC SUSCEPTIBILITIES

	RCy	OCy
	Differences	
$\Delta\chi_m(D_0)$	102×10^{-5} c.g.s. units	141×10^{-5} c.g.s. units
$\Delta\chi_m(D_1)$	241×10^{-5} c.g.s. units	296×10^{-5} c.g.s. units
$\Delta\chi_m(D_2)$	27×10^{-5} c.g.s. units	66×10^{-5} c.g.s. units
	Slopes	
δ_1	7.0 dynes g. (H_2O)/g. (Prot.)	8.3 dynes g. (H_2O)/g. (Prot.)
δ_2	5.4 dynes g. (H_2O)/g. (Prot.)	7.1 dynes g. (H_2O)/g. (Prot.)
$\delta_1 - \delta_{H_2O}$	3.0 dynes g. (H_2O)/g. (Prot.)	4.3 dynes g. (H_2O)/g. (Prot.)
$\delta_2 - \delta_{H_2O}$	1.4 dynes g. (H_2O)/g. (Prot.)	3.1 dynes g. (H_2O)/g. (Prot.)
	Absolute values	
χ(solution) = χ_s	29×10^{-6}[a,b] (0.83)[c]	325×10^{-6}[a,b] (2.82)[c]
$\chi(0) = [\chi_s + \Delta\chi_m(D_1)]$	270×10^{-6}[a,b] (2.58)[c]	621×10^{-6}[a,b] (3.91)[c]
χ(dehydrated 27°) = $[\chi_s + \Delta\chi_m(D_1) - \Delta\chi_m(D_0)]$	168×10^{-6}[a,b] (2.03)[c]	480×10^{-6}[a,b] (3.44)[c]
χ(lyophilized ?) = $[\chi_s + \Delta\chi_m(D_1) + \Delta\chi_m(D_2)]$	297×10^{-6}[a,b] (2.70)[c]	687×10^{-6}[a,b] (4.11)[c]

[a] J. S. Griffith,[3] corrected by addition of 29×10^{-7} temperature-independent term due to polarization of the 3d electrons by the magnetic field. [b] Values from H. Theorell[1] at 22°, averaged and corrected for polarization but not corrected for temperature. [c] Paramagnetic moment μ, calculated in Bohr magnetons using $\mu = \sqrt{\frac{3kT\chi}{N\beta^2}}$; Δ is assumed zero.

the several line segments not having the water value are given in Table I. D_2, not given on the graphs, is the difference in the value of the relative force between the intersection of the lines δ_1 with the force axis and points 0.

Discussion

Since the total increase in diamagnetism on increasing the hydration of trypsinogen is that to be expected from the bound water treated as having the susceptibility value of pure liquid water, it is probable that neither the bound water nor the protein changes intrinsic diamagnetism during hydration from the dry state to highly concentrated solution. On this assumption the contributions of the globin moiety of the cytochrome *c* and the bound water have been subtracted from the measured susceptibilities to give the results of Table I. It is clear that both OCy and RCy show increases in paramagnetism on drying which are to be attributed to the heme group and more specifically to heme iron ions. The present problem is to determine insofar as possible in the absence of data from electron-spin-resonance experiments just how the paramagnetism is to be divided up between spin and orbital contributions. The essential and most general symmetry of the ligand-field situation involved is tetragonal since in most haem complexes the tetragonal perturbation by the porphyrin nitrogens is more important than the octahedral perturbation which includes the fields of the fifth- and sixth position ligands. It is the relative magnitude of the fields of tetragonal and octahedral symmetry, the latter including the out-of-plane ligands, which determines the relative energies of the levels in the D_4 representation. Except in very close approach of nitrogen atoms of the imidazole base groups to the iron ion, so that the symmetry is truly octahedral, we may expect the tetragonal field effects to dominate the energy and to split the degenerate orbital states of the tetragonal representation. As a result, the quenching of orbital contributions to the paramagnetism, except in the octahedral case of close-approach just mentioned, should be nearly complete. Even in the solution state of OCy in which the fields due to the fifth- and sixth-position nitrogens must be large, the orbital contribution of 1.09 magnetons as given by Theorell appears large when compared to that of simple ferric complexes[7] or with other susceptibility results on hemoproteins.[8] It does not seem unreasonable, therefore, to interpret the data of Table I in terms of the number of unpaired electrons, treating orbital contributions as minor. We shall also assume that π bonding with the fifth- and sixth-position has a small effect on the energy though a thorough theoretical treatment of the problem, which does not seem possible now, will certainly have to take π-bonding into account.

We shall consider that the two imidazole groups perturb the iron orbitals about equally through their ligand fields. In reality, this is probably not always true and we must expect to find a strong *trans*-effect existing between these ligands which will vary to produce complicated and diverse effects through changes in bond lengths or angles or through changes in symmetry. Gibson, Ingram and Schonland[9] have already noted the control over the symmetry and the spin *g* factors which the positions of the out-of-plane ligands provide.

To simplify the presentation here, we shall avoid the tetragonal picture and use the more familiar one-electron treatment of the orbitals of the octahedral representation. The spin contributions to the susceptibility are determined by the sorting of the electrons into the three t_{2g} and two e_g d orbitals of this representation. Since the porphyrin plane forces a nearly constant geometry on the four bonds from the porphyrin nitrogen atoms, we may focus attention on the e orbital d_{z^2} which interacts most strongly with the adjust-

(7) P. W. Selwood, "Magnetochemistry," Interscience Publishers, Inc., New York, N. Y., 1956.

(8) R. J. P. Williams, "The Enzymes," Eds. P. Boyer, H. Lardy and K. Myrback, Vol. I, 2nd Ed., Academic Press, Inc., New York, N. Y., 1959.

(9) J. F. Gibson, D. J. E. Ingram and D. Schonland, *Discussions Faraday Soc.*, **48**, 72 (1958).

able imidazole ligands. The probable situation as regards the 3d orbital energy levels, which is the important consideration here, according to Griffith[10] and McClure,[11] is shown in Fig. 4. The three possible spin states for ferric complexes are shown. The energy center has been adjusted to be the same in the three cases but it is the energy differences between the orbitals relative to an almost fixed pairing energy which determines the number of paired spins. A very similar diagram with one additional electron added to a d_{xz} or d_{yz} level is thought to be appropriate for ferrous complexes. The unusually low position of the d_{xy} orbital is attributed to a raising of the d_{xz} and d_{yz} orbitals by the field of the p electrons on the imidazole nitrogens.[10]

The increase in susceptibility on drying of RCy can only be attributed to the unpairing of two or four electrons. By analogy, a similar conclusion may also be drawn tentatively for OCy since the increase in paramagnetism is certainly too large to be due to a decrease in quenching of the orbital contribution on drying. For RCy the lyophilized protein must be either in a state with 2 unpaired electrons (2.82 magnetons) or a mixture of 0 and 4 unpaired electrons. On the basis of Fig. 4, the first alternative would appear the more attractive since $d_{x^2-y^2}$ lies for the most part in the porphyrin plane and would not be expected to interact strongly with the fields of the fifth- and sixth-position ligands. Its relative energy position is, however, dependent on the ratio of the tetragonal and octahedral perturbations and we cannot at present entirely eliminate the $S = 4/2$ state approximated by the left-hand diagram of Fig. 4.

The normal solution state is that on the right of Fig. 4, the d_{z^2} orbital having been raised by the strong ligand-fields of the imidazole nitrogens. In our present interpretation on drying one or both of the imidazole groups are twisted or drawn away from the iron ion by the remainder of the protein so that d_{z^2} drops in energy to provide the middle situation of Fig. 4 with two unpaired electrons.

The same choices are available to us in OCy and the same decisions must be made. The dissolved protein has 1 unpaired electron or, more probably, it is a mixture of forms with 1 and 3 or 1 and 5 unpaired electrons. On drying, we again suggest that in the presence of the strong tetragonal field of the nitrogen atoms of the porphyrin and a protein-controlled reduction of the effects of fifth- and sixth-position ligands the molecules go over into the state with 3 unpaired electrons. Perhaps the new state also has a small residual orbital contribution but our absolute values are too uncertain to support any conclusion as to this point.

With tetragonal symmetry, the state of $S = 3/2$ is possible in ferric complexes and the complex ion ferric porphyrin chloride is a well-substantiated example of this rare occurrence.[10] These states, unusual in small complex ions may be fairly common in haemproteins and other metal com-

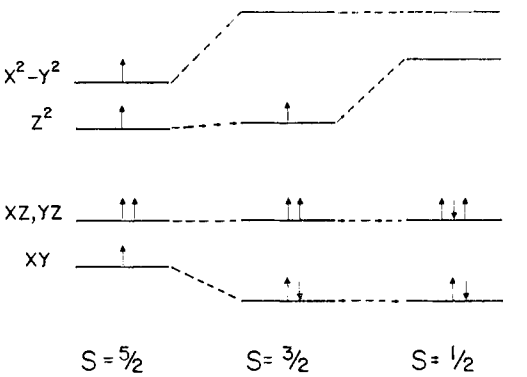

Fig. 4.—The relative energy differences of the 3 d orbitals in the octahedral representation for three spin states.

plexes involving proteins since the protein can enforce on the protein-bound ligands a geometry of varying symmetry and with varying ligand field at the metal ion without changing the kind of liganding groups. Thus the different oxidation–reduction potentials of some of the different cytochromes may be due not to differences in the nature of the fifth- and sixth-position ligands but to a different geometry forced on these ligands by the various globin conformations.[8]

We note also the possibility that octahedral symmetry may be further disrupted and the ligand-field picture further complicated by partial withdrawal of the iron ion from the center of the porphyrin as well as by a tilting of the molecular axes. Both of these situations might be produced by appropriate positioning of the fifth- and sixth-position ligands under protein control, particularly if one of these ligands exerts a strong field and the other a weak field.

In the present picture, any influence which alters the protein conformation to the extent that the position or orientation of the fifth- and sixth-position ligands become changed will alter the electronic properties of the heme-group. It is not difficult to envision the evolutionary process through which required electronic situations are achieved by mutation of the amino acid sequence of hemoproteins. Furthermore, influences *in vivo* which alter protein conformation may alter physiological function. For example, the composition of the effective solvent and the steric situation of cytochrome *c* in the mitochondrian may alter its *in vivo* oxidation-reduction potential in both steric and dynamic ways. It is to be expected then that at least in some cases, detectable alterations in protein conformation should be produced by changes in the electronic properties of the iron produced by oxidation or by change of non-protein ligand in the sixth position. There is evidence from chromatographic studies[12] and from investigations of the effect of proteolytic enzymes on OCy and RCy[13] which indicates a difference in the globin parts of these molecules. It now also appears that many of the observations on the chemical and physical behavior of hemoglobin and

(10) J. S. Griffith, *Discussions Faraday Soc.*, **48**, 81 (1958).

(11) D. McClure, "Brookhaven Symposium, Radiation Research," Suppl. 2, 1960.

(12) R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949.

(13) M. Nozaki, H. Mizushima, T. Horio and K. Okunuki, *J. Biochem.* (*Tokyo*), **65**, 815 (1958).

myoglobin can be explained as aspects of simultaneous changes in protein conformation and heme electronic states on changing sixth-position ligands.[14]

Our opinion that the intermediate spin states appear in the dry cytochromes *c* must be tested for OCy by electron spin resonance studies. Some further justification for it might be provided by the values of the slopes of lines δ_1 and δ_2 (Table I) but there are too many parameters which must be fixed arbitrarily to make any calculations of these slopes from theory meaningful.

Quite apart from the analysis of the spin states is the interpretation of the two kinds of drying experiments: lyophilization at 0° and dehydration at 27°. It is quite clear that the state of the protein is different at the end of these drying procedures but it may be that this difference is not simply a consequence of altered positions of the imidazolium groups but rather may involve a substitution of a water molecule for one or both of these groups. Our remarks above appear to be appropriate for the lines δ_1 in both cases, since there is no evidence that either group has been displaced by a water molecule. We cannot exclude the possibility that one or both imidazole groups have been displaced by other suitable liganding groups in the protein but in view of the lack of any evidence for this situation, it will be ignored here.

The lines δ_2 are quite another matter since near dryness the protein contains one or two very difficultly removed water molecules and the results with RCy suggest the possibility that had these been removed, the susceptibility would have returned to its value in the lyophilized state. If this is true, the states separated by D_0, the initial lyophilized material, or $D_0 + D_2$, the extrapolated initial state after lyophilization, are conformational isomers in which imidazolium groups have been replaced by water molecules. There appears to be no other good reason why such a small number of water molecules should be extractable with so much more difficulty than the others. It is unlikely however that the fifth- and sixth-position ligands in the samples dried at 27° can both be water molecules for in this case, even if the normal position of a liganding water molecule is badly distorted by the nearby protein, complete unpairing of four or five d electrons is to be expected.

It is perhaps of some significance that the two drying procedures do not give identical protein states. This finding supports those of Hnojewyj and Reyerson previously mentioned. Relaxation from both of these states is linear in the amount of water bound up to some point such as C of Fig. 2 and is non-linear in bound water from that point to saturation. The nature of the change near the point C is uncertain. It might be the point at which water molecules begin to displace imidazole groups but if this were true, the fifth- and sixth-position ligands in normal solution could not both be imidazole groups and the total allowable coupling of spins which is observed would not be understandable. Hnojewyj and Reyerson in their studies of the hydration behavior of proteins have observed that the effective monolayer for first coverage includes all the hydrogen bonding valences of the protein except for small groups or cores which do not exchange protons with the solvent. The latter experiments were extended by allowing a protonated protein to exchange with D_2O vapor and the extent of displacement of protons by deuterons measured. The number of exchangeable protons is very nearly equivalent to the number of sites for water binding in the several proteins studied. Although the stoichiometry is a puzzle, the two kinds of experiments suggest that much of the first water molecules bound to a dry protein are bound inside the protein at hydrogen-bonding valences not normally occupied by water in the true solution state. If this is the case, then at some point the "inner" water molecules must be displaced to the outside of the protein since on a time average in solution no great number is expected to remain at any internal point. This extrusion of water would involve a major conformation change and if, as we here suggest, point C is the approximate point at which this change begins to occur, both the sharpness of the change at C for OCy and the slowness of the relaxation at high vapor pressure for RCy could be due to this conformation change. Thus far, no anomaly in the absorption isotherms in the neighborhood of point C has been observed. Although a break in the isotherms might be expected, our information about the hydration process for proteins is too scant to establish any significance for the absence of such a break.

(14) R. Lumry, *Nature*, to be published.

Experimental

OCy, horse-heart preparation Type-III purchased from Sigma Chemical Company (lot 40-B-791), was totally oxidized with ferricyanide and then desalted by dialyzing in a 1% solution to 2.5×10^{-5} *M* KCl equivalent in salt. RCy was formed from this preparation of OCy by reduction with hydrogen over a platinum catalyst. No buffer other than the protein itself was employed. Trypsinogen was a thrice crystallized preparation which had been freed of traces of trypsin by the method of Liener, *et al.*[15]

Purity of the cytochrome solutions was based on measurements of spectra before and after susceptibility experiments. OCy contained less than 1% of RCy and *vice versa*. The spectra of solutions of the two proteins were unchanged following the experiments on susceptibility except for one OCy sample which was heated to 58° in an attempt to remove the last molecule of water. This sample had an unusual spectrum and was only partially soluble. All experiments were carried out at 27 ± 0.1° in the absence of oxygen using a susceptibility balance of the Faraday type in which the sample is enclosed in closed system which allows close control of the composition and temperature of the gas phase. This apparatus has been described elsewhere.[16] Samples were first dehydrated to constant weight in a vacuum (10^{-6} mm.). In non-coenzymic proteins such as trypsinogen, ribonuclease, β-lactoglobulin, lysozyme, etc., the studies of Reyerson and Hnojewyj[5] suggest that no water remains on the polypeptide after this treatment but this situation has not been established for the cytochrome. The dry protein is brought to equilibrium with increasing partial pressures of water vapor. The amount of water bound and the force on the sample produced by the magnetic field were determined by displacement of a reference point on a calibrated quartz spring. The dry protein samples weighed about 0.05 g. and the precision of weight measurements was 3×10^{-3} dynes. The deviations from the drawn lines are

(15) I. Liener, *et al.*, *Arch. Biochem. Biophys.*, **88**, 216 (1960).

(16) A. Solbakken and L. H. Reyerson, *J. Phys. Chem.*, **83**, 1622 (1959).

from 1 to 4 $\times$ 10^{-3} dynes and thus not significant. Precision in bound water determination varied from 1 to 5% depending on the amount of water bound. The field factor for the balance, $H\left(\frac{\partial H}{\partial S}\right)$, was 5.41 $\times$ 10^6 gauss^2/cm.

NOTE ADDED IN PROOF.—Using the auto-oxidation and carbon-monoxide-binding tests the OCy preparations were found to contain 95% native form. The oxidation–reduction titration behavior was that to be expected for a one-electron process and the standard half-cell potential had the accepted value. Although no tests for the presence of "modified" form after the hydration experiments were made, the complete reproducibility of the susceptibility experiments made on the same sample indicates that little modified form was produced by drying and rehydration.

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Solvent Effects on $n \rightarrow \sigma^*$ Transitions; Complex Formation Between Amines and Halomethanes

BY D. P. STEVENSON AND G. M. COPPINGER

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By means of measurements of the ultraviolet absorption spectra of isoöctane solutions of triethylamine with added, (1) chloroform, (2) fluorotrichloromethane, (3) carbon tetrachloride and (4) bromotrichloromethane, it is shown that amines (B) form complexes with halomethanes, (A), that are either 1:1 charge transfer complexes or interact (in 1:1 pairs) to give rise to so-called contact charge-transfer spectra. The complex formation (or contact interaction) accounts for the photochemical instability of solutions of aliphatic amines in carbon tetrachloride solution. The photo reactivities of the amines in the three tetrahalomethanes in the ultraviolet increase in the order, $FCCl_3 < ClCCl_3 < BrCCl_3$.

In connection with our survey of the use of solvent effects on the spectral location of $n \rightarrow \sigma^*$ transitions as means of studying specific solvation of the hydrogen bonding type, it seemed to be of interest to compare by this technique the hydrogen bonding power of chloroform toward amines with that of the previously studied water[1] and simple alcohol[1b] solvent systems. To this end we undertook the comparison of the ultraviolet absorption spectrum of triethylamine in chloroform solution with that of this base in water, isooctane and diethyl ether. In line with out previous experience, we expected to find a blue shift of the chloroform solution spectrum of the ethylamine relative to that of the ether or isooctane spectrum only slightly less than the blue shift that is found in tertiary butyl alcohol solution.[1b] As may be seen in Fig. 1, curves VI and V, respectively, the chloroform solution spectrum shows a large red shift relative to the isooctane solution spectrum, in complete contradiction to our expectation.

This observation immediately suggested the existence of an interaction between the amine and chloroform that is quite independent of any hydrogen bond complexing that these molecules may undergo. As may be seen in curves VII and VIII, the spectra of solutions of triethylamine in fluorotrichloromethane and in carbon tetrachloride show even larger red shifts from the isooctane solution spectrum than does that of the chloroform solution. Interaction here cannot involve hydrogen bonding by solvent. This spectral behavior of triethylamine in the halomethane solutions is that which would be expected if the amine forms a complex with the halomethanes of the type that have become known as "charge transfer complexes."[2,3,4]

In the following paragraphs we will present spectroscopic evidence that triethylamine (B) does form a one to one molecular complex with each of the halomethanes (A), chloroform, fluorotrichloromethane, carbon tetrachloride and bromotrichloromethane, of the type AB. We will also cite photochemical evidence that shows such, AB, complex formation is not limited to B = trialkylamine and that the electronic absorption spectra of these complexes are indeed very probably of the charge transfer type.

The method employed to establish the existence of triethylamine–halomethane complexes and to determine the stoichiometry of the complexes was the observation of the absorption spectra of dilute isooctane solutions containing different ratios of amine to halomethane. In concentration units of moles/liter, the ratio B/A of the solutions measured were approximately 0.75/0.25; 0.50/0.50; 0.25/0.75 and 0.25/0.25. Through the use of calibrated quartz inserts in the 1 cm. cells it was

(1) (a) D. P. Stevenson, G. M. Coppinger and J. W. Forbes, *J. Am. Chem. Soc.*, **83**, 4350 (1961); (b) D. P. Stevenson, *ibid.*, to be submitted for publication.

(2) H. A. Benesi and J. H. Hildebrand, *ibid.*, **71**, 2703 (1949).

(3) R. S. Mulliken, *J. Chem. Phys.*, **19**, 514 (1951).

(4) The reviewer of this paper has suggested that the data to be presented below are better interpreted as indicating that amines and the halomethanes undergo "contact interaction," of the type described by L. E. Orgel and R. S. Mulliken [*J. Am. Chem. Soc.*, **79**, 4839 (1957)] to give rise to the new absorption bands as contact charge-transfer spectra, rather than as actual charge-transfer complexes with finite formation constants. The authors believe their experimental data are adequate to establish the stoichiometry of the interaction, 1:1, be it ordinary charge-transfer complex formation or a case of contact interaction. However, they also feel that it requires over interpretation of the present data to reach a conclusion with respect to the question, is the formation constant of the "complex" very small, but finite, or identically zero? The authors feel that the important aspect of the present paper is their evidence that the halomethanes constitute a hitherto unrecognized class of "acceptors" (albeit weak) for strong donors of the amine type. It should be noted in this connection that interaction of the type reported here is either non-existent or hardly observable in the case of the diethyl ether–carbon tetrachloride system (unpublished observations of one of the present authors (DPS)).