glycine, as determined on the amino acid analyzer[15] (cf. Table I). It is seen that a rapid reaction occurs leading ultimately to reaction of almost all the carboxyl groups in the molecule. Since the presence of exposed, buried, and partially buried groups in proteins is well known, the rather high yields of free carboxyl groups is somewhat surprising, indicating that most of the carboxyl groups in these proteins are on the "surface."

Table I. Extent of Reaction of Protein Carboxyls with BDC and Glycine Methyl Ester

	Added glycine residues found[a]			
Protein	5-min reaction	60-min reaction	5–6 hr reaction	Total free carboxyls in native enzyme
Lysozyme	2.1	4.7	8.1	11[c]
Chymotrypsin	6.2	11.8	15.5	17[d]
Trypsin	4.6	8.8	12.5[b]	11[e]

[a] Per mole of enzyme after acid hydrolysis (total residues minus glycine residues in native enzyme). [b] The slight excess of the glycine value over number of carboxyls in the tentative sequence of Neurath and co-workers may arise from (i) error in determination of glycine (about ±0.5 residue), (ii) autolysis of trypsin, (iii) variation between batches of trypsin, or (iv) the tentative nature of the sequence. [c] R. E. Canfield, J. Biol. Chem., 238, 2698 (1963). [d] B. S. Hartley, Nature, 201, 1285 (1964). [e] K. A. Walsh and H. Neurath, Proc. Natl. Acad. Sci. U. S., 52, 884 (1964).

No products of side reactions which survived acid hydrolysis of the protein were obtained. However, a slow reaction of BDC with phenol to produce O-aryl-isourea was observed on prolonged reaction, and therefore a slow reaction with tyrosine is possible.

The versatility of the procedure is particularly valuable, since it is often desirable to produce parallel modifications introducing groups of varying charge, size, or chemical properties into the same protein. For example, introduction of a chromophoric group can yield information on conformation changes[16] and specificity[17] in enzymes, and a suitable chromophoric modifying reagent, nitrotyrosine ethyl ester, has been obtained by nitration[18] of L-tyrosine, followed by esterfication in ethanol saturated with HCl. The use of 0.10 M nitrotyrosine ethyl ester in place of glycine methyl ester in the above procedure for protein modification yielded a yellow lysozyme derivative containing 1.4 nitrotyrosine ester groups per molecule, which could be degraded to yield several yellow peptides. Glycinamide and hydroxylamine have also been used as modifying reagents. Studies of further variations in the structure of the carbodiimide and the modifying reagent are now being pursued to ascertain the role of carboxyl groups in these enzymes and in other biologically active proteins.

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Formation of Hot OH Bonds in the Radiationless Relaxations of Excited Rare Earth Ions in Aqueous Solutions

Sir:

Enhancement of the luminescence of rare earth ions in aqueous solutions by substituting the water by heavy water has been reported by Kropp and Windsor.[1] Earlier, Hutchinson and Magnum[2] and Wright, Frosch, and Robinson[3] reported increased lifetimes and phosphorescence yields in deuterated hydrocarbons in their triplet state, and offered a theory to explain nonradiative energy transfer which results in radiationless relaxations.[4]

Radiationless relaxation processes may involve three types of initial rate-controlling steps: energy transfer to a single vibrational mode, which is excited to a high vibrational state; energy transfer to several vibrational modes of one molecule; or energy transfer to vibrational modes of several molecules.

We wish to present evidence that at least in aqueous solutions of some rare earth ions the electronic energy of the excited rare earth ion is transferred to a single vibrational mode which is excited to a high vibrational state.

The rare earth ion–D_2O–H_2O system offers several advantages in radiationless deexcitation studies. The ions are readily excited to metastable states which involve only nonbonding "f" electrons. In the D_2O–H_2O solvent, the introduction of the quencher (DHO or H_2O) does not modify the location of the spectral lines, the absorbance, or the chemical properties. Most important of all, the excellent work of Kropp and Windsor indicates that the "major quenching of a rare earth ion in solution is due to hydrogen vibrations about it."

The quantum yield of luminescence of the solvated rare earth ion may be expressed as

$$y = k_r/(k_r + k_h[H]^n + k_d[D]^m + \Sigma k_i[Q_i]) \quad (1)$$

where k_r is the rate of the radiative decay, k_h and k_d are quenching rates due to vibrations involving hydrogen and deuterium, and k_i is the quenching rate due to the presence of the ith impurity. [H], [D], and [Q_i] refer to the respective concentrations; n and m represent the number of hydrogen or deuterium atoms involved in the quenching process.

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Table I

Ion	No. of "f" electrons	Trans.	λ, A	Slope, % H_2O^{-1}	Intercept	Std dev of fit	Correlation coeff	I_{D_2O}/I_{H_2O} Calcd[a]	I_{D_2O}/I_{H_2O} Exptl
Sm^{3+}	5	$^4G_{5/2} \rightarrow {}^6H_{7/2}$	5956	0.217 ± 0.004	1.015 ± 0.013	0.022	0.9991	21.7 ± 0.4	26 ± 3
Eu^{3+}	6	$^5D_0 \rightarrow {}^7F_1$	5916	0.435 ± 0.003	1.007 ± 0.009	0.015	0.9999	43.5 ± 0.3	54 ± 6
Tb^{3+}	8	$^5D_4 \rightarrow {}^7F_3$	5443	0.126 ± 0.003	0.988 ± 0.012	0.018	0.9988	12.6 ± 0.3	12.2 ± 1.5
Dy^{3+}	9	$^4F_{9/2} \rightarrow {}^6H_{11/2}$	5741	0.192 ± 0.005	1.014 ± 0.023	0.039	0.9970	19.2 ± 0.5	18.5 ± 2.0

[a] Assuming $k_r + \Sigma k_i[Q]_i \gg k_d[D]^m$.

The ratio of the emittance of a solution free of hydrogen to the emittance of a solution containing hydrogen is, from eq 1

$$I_0/I = y_0/y = 1 + \{k_h/(k_r + k_d[D]^m +$$

$$\Sigma k_i[Q_i])\}[H]^n \quad (2)$$

The number of hydrogen atoms involved in the quenching process may be determined by measuring the change in I_0/I when small amounts of an H_2O solution of the rare earth ion are added, keeping the deuterium concentration practically unchanged. If $k_r + \Sigma k_i[Q_i] \gg k_d[D]^m$, the coefficient of $[H]^n$ is constant throughout the whole concentration range. If, in addition, $n = 1$, a linear relation is expected for the hydrogen-induced radiationless deexcitation.

Measurements done on 0.1 M rare earth chloride solutions at 23.5 ± 3.0°[5] over the 0–10% H_2O concentration range indicate that within an extremely high confidence limit the relationship between I_0/I and [H] is linear, in agreement with previous results.[1,6] Replotting a reported I vs. % H_2O curve[6] as I_0/I vs. % H_2O (Figure 1), we find a linear relationship over the whole 0–100% H_2O range.[7] The experimental data and their analyses are summarized in Figure 1 and Table I.

The strict linearity at low H_2O concentrations implies that the radiationless relaxations studied involve only a single hydrogen atom (eq 2) and result in the formation of some highly excited hydrogen-containing species. The continuation of the linear relationship over the whole 0–100% H_2O concentration range[1,6,7] (indicated in the last two columns of Table I and in Figure 1) suggests that the hydrogen-containing species is an OH bond.[8] Had a DHO molecule as a whole been required in the rate-controlling step, a maximum in I_0/I would occur at 50% H_2O. HDO·nD_2O requires a maximum between 0 and 50% H_2O. Other species like H_2O, $H_2O·nD_2O$, and aggregates containing more than one hydrogen require a quadratic or higher power dependence. This leaves the OH bond as the only acceptable species.

The sensitivity of the present measurements to a possible higher order dependence of I_0/I on the OH

concentration has been tested by attempting a least-squares fit with $n \geq 2$ (eq 2). At low H_2O concentrations, the fit becomes extremely poor in all the four rare earth ions. At high H_2O concentrations, the deviations become unacceptable.
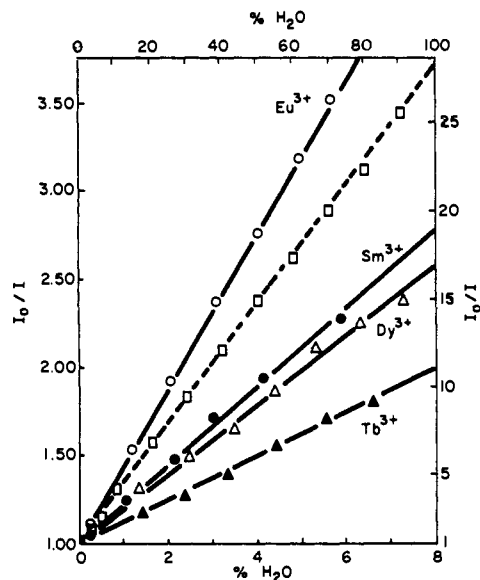

Figure 1. Quenching of the luminescence of rare earth ions in D_2O. Top and right scales refer to dashed line, which is a replot of Gallagher's[6] I vs. % H_2O curve for europium as I_0/I vs. % H_2O.

Using a value of 3600 cm^{-1} for the fundamental OH vibration, we find that the changes in the vibrational quantum numbers which are required to bridge the smallest gaps between the excited and ground multiplets[9] are $\Delta v \geq 5$ for terbium, $\Delta v \geq 4$ for europium, $\Delta v \geq 2$ for samarium, and $\Delta v \geq 2$ for dysprosium.

These results, along with previous data on radiationless relaxations in gases,[10] indicate that vibrational transitions in the energy acceptor are not necessarily subject to optical selection rules; energy may be transferred to a single bond which is excited to a high vibrational state.

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(5) The measurements were temperature independent over this range. This could be expected from the reported invariance of lifetimes over ranges of 100°.[1]

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(7) The standard deviation of fit is 1.9% and the correlation coefficient for 13 points 0.9997.

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