Bentley and Humphreys.[7] The specific surface area was only about 0.2 m^2/g, or about the geometric expectation, and the isotherm, while not accurately determinable, appeared similar to that of run 4b. A second sample of 11.3 g of day-old Denver snow, collected on Jan 18, 1966, gave reproducible isotherms corresponding to the very low c value of unity and a Σ of 0.4 m^2/g. Surface contamination from adsorption of atmospheric impurities cannot be excluded in this case, however.

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A Procedure for the Selective Modification of Carboxyl Groups in Proteins

Sir:

The chemical modification of amino acid residues provides essential information in the correlation of protein structure and function. There is at present no simple published procedure for producing well-characterized modifications of carboxyl groups which is capable of general application to proteins. Methanolic solutions of HCl can give good yields of methyl esters, but the high methanol and HCl concentrations limit their use to the most stable proteins.[1,2] Much milder conditions may be used with diazo compounds, but the instability of these reagents in aqueous solution severely limits the yields of esters which can be obtained.[3–8] Carbodiimides have also been used[9–12] as modifying reagents under mild conditions, but the products are poorly characterized, or the yields are low.

We have found that a procedure involving a water-soluble carbodiimide and a modifying reagent can lead to rapid and quantitative modification of carboxyl groups under mild conditions. The basic reaction is shown in eq 1, in which the carboxyl group is activated by the carbodiimide in the first step, presumably by the mechanism suggested by Khorana.[13] The nucleophilic modifying reagent, HX, then attacks the activated carboxyl group to yield the products. A typical procedure involves N-benzyl-N′-3-dimethylaminopropylcarbodiimide (BDC) as the activating carbodiimide and glycine

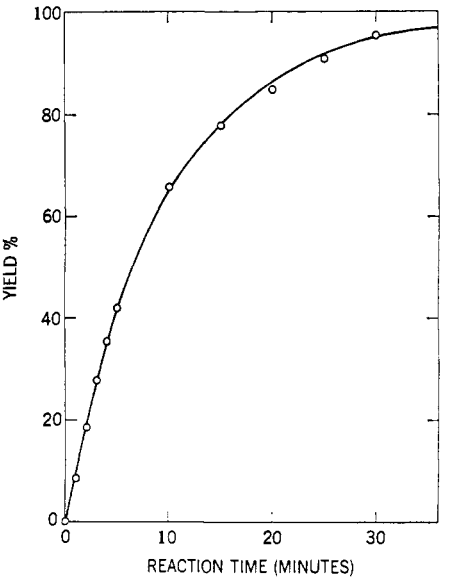
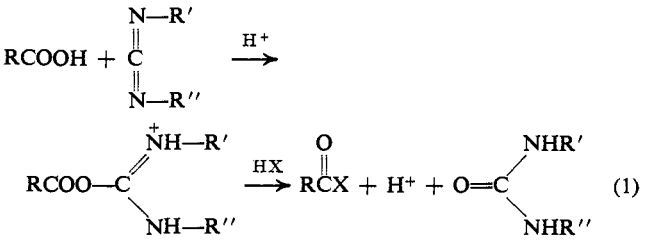


Figure 1. The yield of methyl m-nitrohippurate from the reaction of m-nitrobenzoic acid (5×10^{-3} M) and glycine methyl ester (1.0 M) with BDC (0.10 M) at 25° and pH 4.75 maintained with a pH-Stat.



methyl ester as the modifying reagent. BDC was synthesized from benzyl isocyanate and N,N-dimethyl-1,3-propanediamine by the method of Sheehan and coworkers.[14] It was crystallized from methylene dichloride–ether mixtures as its p-toluenesulfonate, mp 118–119°). *Anal.* Calcd: C, 61.7; H, 6.94; N, 10.80; S, 8.23. Found: C, 61.7; H, 6.79; N, 11.03; S, 8.37.

Kinetic studies with simple carboxylic acids led to conditions giving quantitative yields, for example, the reaction of m-nitrobenzoic acid (5×10^{-3} M), glycine methyl ester (1.0 M), and BDC (0.10 M). Aliquots were quenched with ten volumes of 1.0 M acetate buffer at pH 4.75, acidified with 4 M HCl, extracted with chloroform, and the m-nitrobenzoic acid back-extracted into carbonate buffer at pH 9.5. The product, methyl m-nitrohippurate, was estimated from its absorption at 255 mμ in the chloroform layer, and a graph of yield *vs.* time is shown in Figure 1, with a theoretical first-order curve calculated from $k = 1.5 \times 10^{-3}$ sec^{-1} and an ultimate yield of 100%.

To test this procedure on protein carboxyl groups, the enzymes lysozyme, chymotrypsin, and trypsin (at 10 mg/ml) were allowed to react with 1.0 M glycine methyl ester hydrochloride and 0.10 M BDC at 25° and pH 4.75 in a pH-Stat. The reaction mixture was quenched by dilution with ten volumes of 1.0 M acetate buffer at pH 4.75 and extensively dialyzed. The number of glycine methyl ester residues introduced per mole of enzyme was then calculated from the additional covalently bound

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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-arylisourea 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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