of DTT and 436, 490, 48.4, 34.6, and 15.5, respectively, in the presence of 20 m*M* DTT.

7. For kinetic studies, tissue homogenates were incubated with various doses of T_4 for 15 minutes at 37°C in the absence and in the presence of DTT, and the data on the amount of T_3 produced were examined by using a Lineweaver-Burk plot [H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934)]. The K_m for conversion of T_4 to T_3 by fetal liver homogenate was 5.5 μM in fetuses A and B (Fig. 2) in the absence of DTT and 5.5 and 6.25 μM, respectively, in the presence of 20 m*M* DTT. The V_{max} (nanograms of T_3 per gram equivalent of tissue per hour) was 288 in fetus A and 330 in fetus B in the absence of DTT and 2310 and 3078, respectively, in the presence of 20 m*M* DTT. The K_m for conversion of T_4 to T_3 by maternal liver homogenate (Fig. 2) was 1.5 μM in the absence and 2.5 μM in the presence of 20 m*M* DTT, whereas V_{max} (ng/g-eq hour) was 576 in the absence and 2881 in the presence of 20 m*M* DTT.
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9. Total and nonprotein sulfhydryl groups were measured by using Ellman's reagent [J. Sedlak and R. H. Lindsay, *Anal. Biochem.* **25**, 192 (1968)] in liver homogenates of four fetal sheep (gestational age, 132 to 137 days), four neonatal sheep (2 to 8 hours after delivery), and four maternal sheep. The mean content of total sulfhydryl groups in fetal liver, 16.7 ± 2.3 mmole/kg (wet weight), was significantly ($P < .05$) lower than that (23.2 ± 1.3 mmole/kg) in maternal liver but did not differ significantly from that (19.7 ± 1.8 mmole/kg) in neonatal liver. However, the mean content of nonprotein sulfhydryl groups in fetal liver (2.2 ± 0.40 mmole/kg) was significantly ($P < .005$) less than that in maternal (4.6 ± 0.38 mmole/kg) as well as neonatal (5.5 ± 0.52 mmole/kg) liver.
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Methylation of Humic Acid Fractions

Abstract. *A new three-step permethylation procedure for humic acid fractions has been developed. In the first step, carboxylic acid groups are methylated with diazomethane in dimethylformamide; in the second step, hydroxyl groups are methylated with methyl iodide and dimethyl sulfinyl carbanion in dimethylformamide; and in the third step, acidic groups hydrolyzed in the earlier steps are methylated with diazomethane. The resulting product is completely soluble in methylene chloride (a high degree of methylation has been achieved).*

Humic substances are the most abundant group of organic macromolecules in natural waters and soils. They are also among the most reactive materials in the environment, interacting strongly with practically all of the other organic and inorganic components of natural water systems (*1*).

Traditionally humic substances have been divided into three major fractions—humic acid, fulvic acid, and humin—on the basis of their differing solubilities in basic and acidic solutions. In this report we shall limit our discussion to humic acid.

In spite of their importance in environmental chemistry, very little is known about the chemical structure of the humic acids. In practically all of the studies on structural elucidation of the humic acids, degradation procedures have been used which have yielded only low-molecular-weight fragments (*2*).

Most workers who have studied the chemical structure of humic acids have used the traditional fractionation scheme without attempting to obtain further chemical fractionation of their preparations, even though it is generally recognized that they are mixtures of chemical species. Instrumental techniques such as infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR) have therefore yielded only the most general information, whereas in chemical degradation studies it has been necessary to use reagents which attack a variety of different chemical bonds. It has not been possible to use the highly specific degradation techniques which have been successful in elucidating the chemical structure of other natural products such as polysaccharides, steroids, or proteins because humic acids have proved to be highly resistant to chemical attack by many of the reagents that are used for partial degradation. In addition, unfractionated humic acids are insoluble in most organic solvents. We have suggested (*3*) that this resistance to chemical attack arises from the fact that humic acids form molecular aggregates in solution that must be disaggregated in order that specific functional groups in the humic acid molecules be exposed to chemical reaction.

Our approach to the elucidation of the chemical structure of humic acids is based upon a model we have proposed elsewhere (*3*). In this model humic acids are depicted as being made up of molecular aggregates that are held together by noncovalent and weak covalent bonds. In order to determine the chemical structure for humic acid, it is necessary to disrupt this aggregation and to isolate the various chemical species. The first step in this approach is the fractionation of natural humic acids by adsorption chromatography on Sephadex (*4, 5*). We have shown that fractionation breaks up "mixed aggregates" that are composed of several different chemical species. The next step consists of the elimination of hydrogen bonding with a concomitant reduction in aggregation. This reduction of hydrogen bonding is accomplished by replacing the protons in acidic, phenolic, and alcoholic groups with methyl groups. After this derivatization, the humic acid fractions are further fractionated and each of the fractions is isolated and examined by conventional instrumental techniques. If necessary, controlled degradation reactions are carried out on each of the fractions.

A wide variety of different methylation procedures has been tried on humic acid preparations (*6, 7*). In practically all of the procedures the humic acid is suspended in solvent, and methylation reagents such as dimethyl sulfate and potassium carbonate, methyl iodide and silver oxide, or diazomethane are added to the mixture. These methods have all of the disadvantages inherent in reactions carried out on heterogeneous mixtures. In an earlier paper we reported that humic acid fractions can be dissolved in pyrrolidone and methylated with diazomethane (*7*). Diazomethane will methylate carboxyl groups and some highly acidic phenolic groups; however, weakly acidic phenolic groups and carbohydrate alcoholic groups, which are also probably present in humic acids (*1*), will not react. Therefore, it was necessary to develop another procedure for more complete methylation.

We have found that the methyl esters of humic acid fractions that have been prepared by diazomethane methylation may be further methylated by a modification of the procedure developed by Hakomori (*8*) for the permethylation of saccharides in which methyl sulfinyl carbanion is the base and methyl iodide is the methylating reagent. The methylated humic acid fractions obtained in this way are completely soluble in both methylene chloride and chloroform.

The initial esterification step with diazomethane is necessary because without it the product obtained from the Hakomori reaction is not soluble in methylene chloride or in chloroform. The fact that the methylated products are completely soluble in these solvents indicates that a high degree of methylation has been achieved; NMR and IR studies have provided additional evidence of this methylation.

The permethylation procedure that we have developed is simple and straightforward. The humic acid fractions were isolated as outlined by Wershaw *et al.* (*7*); dissolved oxygen was purged from the solutions with nitrogen during all the steps in which the *p*H was above 8, including the initial extraction of humic

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acid from the soil. All of the humic acid fractions obtained in our fractionation scheme (*4*), with the exception of fractions 1A and 1B which are clay-humic acid complexes, can be methylated by this technique.

In our work with diazomethane (*7*) we dissolved the humic acid fractions in pyrrolidone. We have found, however, that in most instances *N*,*N*-dimethylformamide (DMF) may be substituted for pyrrolidone as solvent for the humic acid fractions. This solvent has the advantage of being easier to remove than pyrrolidone after the reaction. The methylation can then be carried out as described in (*7*) with the following modifications: (i) 0.25 percent (weight-volume) solutions of each humic acid fraction are prepared in redistilled DMF which has been dried over molecular sieve 5A; (ii) after methylation, ether and diazomethane are driven out of the solution by heating in a water bath; the material is completely dried under vacuum, and the dried product is redissolved in DMF (0.25 percent).

Methyl sulfinyl carbanion was prepared as outlined by Corey and Chaykovsky (*9*), and 1 ml of the resulting solution was added to 20 ml of the humic acid solution that had been methylated with diazomethane. An equal amount (1 ml) of methyl iodide that had been distilled over molecular sieve 5A was added, and the mixture was allowed to stand overnight.

At the end of the reaction period 2 ml of redistilled HCl solution (approximately 20 percent HCl) and 20 ml of water were added to the reaction mixture. This solution was then extracted three times with 20-ml aliquots of distilled methylene chloride. After three extractions only a very faint color was observed in the water layer, an indication that essentially all of the methylated humic acid had been extracted into the methylene chloride. The methylene chloride aliquots were combined and exhaustively extracted with water to remove the DMF and other reagents. Any free iodine produced may be removed by washing with a sodium thiosulfate or potassium iodide solution. The methylene chloride solution was then evaporated to dryness in a hot water bath. The product was redissolved in methylene chloride and remethylated with diazomethane in order to methylate any acidic groups resulting from the hydrolysis of esters by the acidification step. The final product was evaporated to dryness in a hot-water bath.

The humic acid derivatives obtained by this procedure have been fractionated by gel permeation chromatography and high-pressure liquid chromatography. Our preliminary examination of these products indicates that this methylation method is a useful approach to the elucidation of the chemical structure of humic acids.

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Inhibition of Carbonic Anhydrase by Anions in the Carbon Dioxide–Bicarbonate System

Abstract. *Chloride and iodide ions exhibit noncompetitive inhibition of carbon dioxide hydration catalyzed by bovine carbonic anhydrase and competitive inhibition of bicarbonate dehydration. Consideration of the net velocity at equilibrium leads to a satisfactory resolution of the dilemma concerning the different concentrations of such anions required to halve the initial reaction rate for each substrate.*

A controversy has arisen in connection with the anionic inhibition of carbonic anhydrase with CO_2 and HCO_3^- as substrates (*1*–*3*). Maren *et al.* (*1*) reported that the values of I_{50} (the concentration of an inhibitor that produces 50 percent of the original activity) in HCO_3^- dehydration were about three times those in CO_2 hydration. Koenig and Brown (*2*) argued from equilibrium considerations that these two sets of I_{50} values should agree and questioned the experimental procedure of Maren *et al.*, especially with regard to the large variation in *p*H accompanying their colorimetric measurements. Maren (*3*) replied that the anions exhibited noncompetitive inhibition of the enzymatic hydration of CO_2 and competitive inhibition of the HCO_3^- dehydration, and that under these conditions the Haldane relation need not have been violated by the different I_{50} values obtained by Maren *et al.*

We thought it desirable to determine independently the nature of the anionic inhibition of carbonic anhydrase, and also to provide a clear-cut explanation of why the addition of inhibitory anions to an equilibrium mixture in the presence of enzyme does not shift the position of equilibrium, if the anions reduce the initial reaction rates from the two substrates to different extents, as Maren *et al.* (*1*) reported. Consequently, we examined the effect of added anions on initial reaction rates in the interconversion of CO_2 and HCO_3^- catalyzed by bovine carbonic anhydrase, using a stopped-flow technique in which the *p*H variation during the monitoring of initial rates can be minimized.

The experimental procedure was similar to that described by Khalifah (*4*), except that experimentally obtained buffer factors were used instead of calculated ones. *N*-Methylimidazole (0.02*M*) and *p*-nitrophenol ($4 \times 10^{-5}M$) were used as a buffer-indicator system. Ionic strength was maintained at 0.1 with Na_2SO_4. Anions were added as their potassium salts. In HCO_3^- dehydration, a calculated small amount of H_2SO_4 was added to the buffer solution with a microsyringe to obtain the correct *p*H on mixing, considering the H_2CO_3-HCO_3^--CO_3^{2-} equilibrium in substrate solutions. The pK_a values of the H_2CO_3-HCO_3^- and HCO_3^--CO_3^{2-} transitions were taken as 3.6 and 10.3, respectively (*5*). Substrate concentrations were 0.0034 to 0.017*M* for CO_2 hydration and 0.01 to 0.05*M* for HCO_3^- dehydration. The concentration of enzyme was $7.8 \times 10^{-8}M$, measured spectrophotometrically and corrected by using ethoxzolamide (*6*).

The initial portion of the reaction was traced by using the Durrum-Gibson stopped-flow apparatus, which was interfaced with the PDP-8L digital computer. The initial *p*H was 7.00, and the maximum *p*H variation during the observation period was less than 0.1. From the

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