The data indicate clearly that the presence of borate ion in the buffer has no effect upon the mobility of serum albumin, while it increases the mobility of orosomucoid about 20% and that of the inhibitor about 40%. On the other hand, quite unexpectedly, the presence of sulfate ion in the buffer has a substantial effect upon the mobilities of all proteins. As far as we are aware, no other studies of the binding of sulfate ions by proteins have been published. Using the charge-mobility relationship of Longsworth and Jacobsen,[5] we estimate that a minimum of about 3 moles of sulfate ion is bound per mole of serum albumin. Dialysis equilibrium studies in which methyl orange was used as a competing ion, do not reveal appreciable binding of sulfate by albumin. However, this method may be too insensitive.[6]

Paper electrophoresis studies[7] have shown that mono- and oligosaccharide derivatives combine strongly with borate when a glycosyl hydroxyl group and an adjacent hydroxyl group are free. However, union of borate with carbohydrate can also occur, though less strongly, when vicinal hydroxyl groups only are available.

In a recent report, Northcote[8] has described the free solution electrophoresis in borate buffer, *p*H 9.2, of neutral polysaccharides and has concluded that complexing with borate occurs with both *trans*- and *cis*-vicinal hydroxyl groups, although more strongly with *cis*- than with *trans*-hydroxyls. Since the comparisons were not made in buffers of the same ionic strength, this conclusion has not been clearly established.

The above data extend these observations to carbohydrate containing proteins and suggest that the carbohydrate moiety of the molecule possesses vicinal hydroxyl groups available for complexing with borate ion. In the case of the inhibitor, such complexing occurs without detectable denaturation.[3] Electrophoresis in borate containing solutions appears to be a procedure of value for electrophoretic separations of mucoproteins. In addition, information as to structure may also be obtained.

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Formation of 3,5-Dinitrobenzoates from Acetals and Ketals with 3,5-Dinitrobenzoyl Chloride[1]

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During some work on a commercial solvent we observed what appeared to be the direct formation of methyl 3,5-dinitrobenzoate from dimethyl acetal and 3,5-dinitrobenzoyl chloride. This suggested the possibility of characterizing the alcohol groups of acetals and ketals as solid 3,5-dinitrobenzoates without preliminary hydrolysis and separation of the alcohol.[2] Earlier, Post[3] had reported 60–70% yields of alkyl benzoates from equimolar quantities of aliphatic acetals and benzoyl chloride allowed to react at reflux temperatures. The second product was believed to be an α-chloroalkyl alkyl ether. Thus, with diethyl acetal the reaction would be

$$CH_3CH(OC_2H_5)_2 + C_6H_5COCl \longrightarrow C_2H_5OOCC_6H_5 + CH_3CH(Cl)OC_2H_5 \quad (1)$$

Previously, zinc iodide had been used to catalyze the similar reaction of dimethyl acetal and benzoyl chloride,[4] but no catalyst is needed.[3] Therefore, the replacement of a single alkoxy group in the acetals by chlorine from benzoyl chloride takes place more readily than the analogous reaction of dialkyl ethers with acid chlorides which requires zinc or ferric chloride.[5]

Ketals of methyl ketones react with benzoyl chloride at 100–140° to give a benzoate ester and, instead of an α-chloroether, an alkyl chloride and the methyl ketone are formed[6]

$$CH_3C(OCH_3)_2R + C_6H_5COCl \longrightarrow CH_3OOCC_6H_5 + CH_3Cl + CH_3COR \quad (2)$$

Esters also have been obtained from acetals and ketals with acids or acid anhydrides but vigorous conditions were required.[7–9] With an acid catalyst, however, methylal and aliphatic acid anhydrides (not aromatic acid anhydrides) at reflux give 86–95% yields of methoxymethyl acetate, etc.[10]

In studying the formation of 3,5-dinitrobenzoates from acetals and ketals with 3,5-dinitrobenzoyl chloride, we considered reactions 1 and 2, and also the possibility that both alkoxy groups might be replaced by chlorine with the formation of an alkylidene chloride, a vinyl chloride or an acetylene, depending upon the extent of dehydrochlorination

$$RCH_2CH(OR')_2 + 2ArCOCl \longrightarrow RCH_2CHCl_2 + 2ArCOOR' \quad (3)$$
$$\downarrow$$
$$RCH{=}CHCl + HCl$$
$$\downarrow$$
$$RC{\equiv}CH + HCl$$

The stoichiometry of the reaction of anhydrous 1,1-dimethoxyethane and 3,5-dinitrobenzoyl chloride was determined at several reactant ratios, times and temperatures. With excess acetal, methyl 3,5-dinitrobenzoate is not formed until the reaction time at reflux (*ca.* 70°) exceeds five minutes. At a 5.5/1 mole ratio and 30 minutes reflux the yield of ester is 53%. Reversing the reactant ratio to excess acid chloride gave mixtures which decomposed at 65°, but at room temperature for 72–120 hours the yields were 45–50%. Thus, under these conditions the ester readily forms through

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the replacement of only one methoxy group (equation 1). The volatile product of this reaction contained no acetylene and consisted solely of α-chloroethyl methyl ether, as shown by its chlorine content and distillation behavior. Quaternary ammonium salts could not be made as derivatives because of dehydrochlorination, but bromination gave the expected α,β-dibromoethyl methyl ether.[11]

Therefore, 1,1-dimethoxyethane reacts with 3,5-dinitrobenzoyl chloride through the replacement of one methoxy group by chlorine to form methyl 3,5-dinitrobenzoate and α-chloroethyl methyl ether, which proves the reaction (equation 1) proposed earlier.[3]

Extending this preparation to other acetals and ketals showed that satisfactory yields of the 3,5-dinitrobenzoate are obtained readily (Table I). Therefore the alcohol part of at least the simpler acetals and ketals derived from aliphatic alcohols can be characterized satisfactorily without hydrolysis and separation of the alcohol.

TABLE I

REACTION OF ACETALS AND KETALS WITH 3,5-DINITROBENZOYL CHLORIDE[a]

Acetal or Ketal	B.p., °C.(mm.)	Heating time at reflux,[b] min.	Yield of recryst. ester,[c] %
Dimethoxymethane[a] (III)	42–43	30	1–2
Dimethoxymethane[a] (III)	...	240	41
1,1-Dimethoxyethane (IV)	63–64	30	53
1,1-Diethoxyethane (V)	100–102	10	75
1,1-Diethoxyethane (V)	...	30	84
Di-*n*-butoxymethane (VI)	177–178	30	85
Phenyldiethoxymethane (VII)	104–106(18) 222(760)	10	55
2,2-Diethoxypropane (VIII)	110–112	10	62
2,2-Di-*n*-butoxypropane (IX)	54–57(0.5) *ca.* 215(760)	30	87
1,1-Diethoxycyclohexane (X)	78–82(18) *ca.* 180(760)	10	70

[a] Mole ratio of 11/1 acetal or ketal to chloride in all experiments except 5.5/1 with III. [b] The oil-bath temperature was held for gentle reflux. [c] In all cases admixture with authentic ester did not depress the melting point. The percentage yield is based on the acid chloride.

The mechanism of the reaction very likely involves electrophilic attack of an oxygen by an aroylcarbonium ion, breaking of the carbon–oxygen bond, and reaction with the chloride ion. On this basis the inertness of the second alkoxyl is attributed to the greater +I effect of Cl compared to OR′ which reduces the reactivity of the oxygen in the α-chloroether toward the aroyl ion. Resonance stabilization of the intermediate assists the replacement reaction, while the corresponding intermediate in the case of dialkyl ethers, which do not react under these conditions, cannot be stabilized by resonance.

Experimental

Reactants.—3,5-Dinitrobenzoyl chloride, m.p. 68–69°, was kept in a desiccator and the melting point checked frequently. When necessary, it was crystallized from *n*-pentane. Commercially available acetals and ketals were carefully purified[12] to obtain anhydrous, alcohol-free compounds by washing with water, drying and fractionating through a glass helix column, 12 mm. in diameter, 43 cm. long. Others were made by the Claisen orthoformic ester synthesis.[13] Boiling points and refractive indexes checked satisfactorily with published values.

1,1-Dimethoxyethane (IV) Plus 3,5-Dinitrobenzoyl Chloride (II).—These experiments were run with 2.0 g. (8.4 millimoles) of II and various quantities of IV at mole ratios, IV/II, of 22/1, 11/1, 5.5/1, 1/2, and 1/4 in all-glass apparatus heated by an oil-bath. At reflux the reactants were at about 70°. After cooling to room temperature, volatile products were removed by suction. Excess II was extracted by crushing the solid residue, mixing well with 30 ml. of 5% sodium carbonate solution, and heating the mixture with stirring at 40–50° for 10 minutes. Crude methyl 3,5-dinitrobenzoate was collected by filtration, washed with water, dried, and crystallized from 95% ethanol. A mixed melting point with authentic ester gave no depression.

Isolation of α-Chloroethyl Methyl Ether.—A larger scale experiment with IV and II was carried out in a flask fitted with two gas inlet tubes and an outlet tube connected to two Dry Ice-cooled traps. These were followed by a gas-washing bottle containing 4 *N* potassium hydroxide solution, an empty trap, and a gas-washing bottle containing aqueous cuprous diammine chloride.[14,15] One inlet tube to the flask extended to the surface of the reactants, the other had a capillary tip reaching to the bottom of the flask. In the flask were placed 4.68 g. (52 millimoles) of IV and 24.0 g. (104 millimoles) of II. While standing at room temperature for 4 hours, a solid crystalline mass formed. Nitrogen gas was passed through the capillary tube and, by alternately evacuating the system and passing nitrogen through, all volatile material was carried to the traps and gas washers. Then 110 ml. of aqueous 5% sodium carbonate was added to the residue, the solid was crushed, and the mixture removed. With an additional 170 ml. of sodium carbonate solution the suspension was heated at 40–50° for 10 minutes. The filtered solid weighed 11.7 g., 50%, m.p. 105–107°. Crystallization from 50 ml. of 95% ethanol gave 9.8 g. (43.4 millimoles), 42%, m.p. 106–107.5°.

The sodium carbonate washings were analyzed for chloride (Volhard) and acidified to precipitate 3,5-dinitrobenzoic acid (XIII). The chloride content equalled 52.6 milliequivalents of hydrogen chloride; 10.6 g. (50.0 millimoles) of XIII was recovered. The filtrate from the crystallization of the methyl ester gave an additional 1.0 g. (4.7 millimoles) of XIII.

The Dry Ice traps contained 4.5 g. of colorless, fuming liquid which smelled strongly of hydrogen chloride. A few drops added to water gave the odor of acetaldehyde; this solution precipitated silver chloride from aqueous silver nitrate. Titration of an aliquot with 0.1 *N* potassium hydroxide showed that the condensate contained or formed 1.57 g. (43.2 millimoles) of hydrogen chloride.

Other aliquots of the condensate analyzed for total chlorine (Carius) contained the equivalent of 1.58 g. of hydrogen chloride. Thus, substantially all of the chlorine was present as hydrogen chloride or an easily hydrolyzed chloride. The potassium hydroxide gas-washing bottle contained 0.018 g. of hydrogen chloride (Volhard).

A portion of the trap condensate distilled at 64–73°, $n^{25}D$ 1.3930; a black, tarry residue remained. Both distillate and residue smelled of hydrogen chloride. The distillate was analyzed for chlorine by titrating with base.

Anal. Calcd. for C_3H_7OCl (α-chloroethyl methyl ether); Cl, 37.5. Found: Cl, 33.0.

The cuprous diammine chloride solution gave no test for acetylene.

α-Chloroethyl methyl ether, made from paraldehyde, methanol and hydrogen chloride,[11,16] was compared with the trap condensate. The purified product (39% yield) distilled at 67–73° ($n^{25}D$ 1.3930), evolved hydrogen chloride, and left a tarry residue. Again, the chlorine content of the distillate was less than calculated.

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Anal. Calcd. for C_3H_7OCl: Cl, 37.5. Found: Cl, 34.8.

Attempts to prepare quaternary ammonium salts as solid derivatives were not successful. Even at 0°, N,N-dimethylbenzylamine, dimethylaniline or pyridine and the chloroether gave only the amine hydrochloride.

α,β-Dibromoethyl Methyl Ether.—A portion of the distilled condensate from the Dry Ice traps was brominated.[11] The yield was 75%, boiling 69–71° (22 mm.).

Anal. Calcd. for $C_3H_6OBr_2$: Br, 73.5. Found: Br, 73.2.

Generalized Procedure.—The directions for making 3,5-dinitrobenzoate derivatives are as follows. A mixture of 1.0 g. of 3,5-dinitrobenzoyl chloride and 2–3 ml. of acetal or ketal in a 25-ml. round-bottom flask is heated by an oil-bath at gentle reflux for 5–60 minutes. The time depends on the reflux temperature—if the acetal boils below 60°, use 60 min. For boiling points between 60 and 100°, 30 minutes is sufficient, and over 100° the reaction requires only 5–10 minutes. If the mixture turns dark, heating should be stopped because the yield at this point will be adequate. After cooling to room temperature, 10 ml. of aqueous 5% sodium carbonate is added, and the mixture is solidified by cooling. This is crushed in a mortar, and an additional 10 ml. of sodium carbonate solution added. After heating in a beaker with stirring at 45–50° for 10 minutes, the crude ester is collected, washed with water, dried in air, and crystallized from 95% ethanol.

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A Test for Enzymatic Transpeptidation Reactions

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In investigations on the action of enzymes on proteins, peptides and amino acids, it is important to decide whether or not transfer of amino acid residues takes place under the conditions of the experiment. This problem can be solved by adding small amounts of radioactive substrates of the respective enzyme, and determining whether the radioactivity is incorporated into the reaction products. In our laboratory this method has been used to investigate the mechanism of plastein formation.

A peptic digest of ovalbumin was prepared according to Tauber.[3,4] Thirty ml. of the neutralized and concentrated digest was placed in each flask and mixed with the substrates shown in Table I. After 36 hours incubation with 9.6 mg. of crystalline chymotrypsin (Armour), at *p*H 7.30 and 37°, the insoluble plastein formed was washed, dried, plated and counted in a gas flow counter. Table I shows that the isolated plastein was radioactive after incubation with phenylalanine ethyl ester, but practically free of activity after incubation with phenylalanine, glycine or glycine ethyl ester. Evidently, the formation of plastein involves transpeptidation, *i.e.*, the transfer of phenylalanyl residues from ethanol to peptides of the peptic digest. This is in agreement with results of Brenner, *et al.*,[5-7] obtained with chymotrypsin.

TABLE I

RADIOACTIVITY OF PLASTEIN FORMED IN THE PRESENCE OF VARIOUS C^{14} SUBSTRATES

Type	Substrates[a] Wt., g	Counts/min.	Plastein Counts/min. per mg.
Glycine	0.50	82,500	0.13
Glycine ethyl ester-HCl	.94	69,500	.22
Phenylalanine	.11	134,000	.10
Phenylalanine ethyl ester-HCl	.14	132,000	28.4 (28.6[b])

[a] Labeled by C^{14} in 2-position. [b] After extraction with acetone.

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(2) Predoctorate fellow of the National Science Foundation, 1952-1954.

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The fact that only traces of glycine ester are incorporated is in accordance with the substrate specificity of chymotrypsin.[8,9] Since the radioactivity of the insoluble material is not extracted by acetone,[10] it cannot be due to contamination by phenylalanylphenylalanine.

Obviously, the method described in the preceding paragraphs also can be used for other enzymes. While the esters of isotopically labeled phenylalanine, tyrosine or methionine are suitable substrates for chymotrypsin, or cathepsin C, labeled lysine or arginine ester or amide would have to be used as test substrates for trypsin or cathepsin B.[11]

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On the $AlCl_3$-catalyzed Reaction between Ethylene Oxide and Malonic Ester

BY HAROLD HART AND OMER E. CURTIS, JR.

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It was claimed recently[1] that malonic ester can be alkylated by ethylene oxide, using anhydrous aluminum chloride, to give a *quantitative* yield of γ-butyrolactone. Because of an interest in lactones as intermediates in dicycloalkyl ketone syntheses[2] and because we were unaware of any phenomenon of "dimorphism" which would cause γ-butyrolactone to have two different boiling points 45° apart, as was claimed,[1] we reinvestigated the reaction.

We have found that the products described by Raha are, in fact, recovered malonic ester and the ester-interchange product, β-chloroethyl ethyl malonate. In addition, a third product, bis-β-chloroethyl malonate, was obtained. We isolated no γ-butyrolactone from the reaction.

Experimental

The "alkylation" was carried out following Raha's procedure identically, and also on a larger scale, except that the ethylene oxide was obtained from a cylinder (Matheson) rather than generated from chlorohydrin. From five moles each of malonic ester, aluminum chloride and ethylene oxide there was obtained, upon distillation through an efficient column, three main fractions: fraction 1, b.p. 60–61° at 1 mm., $n^{25}D$ 1.4130, 504 g.; fraction 2, b.p. 104–105° at 4

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