peak by three experiments. (1) Addition of one mole of mercuric chloride per mole of albumin completely abolishes the 375 mμ band. (2) Addition of one mole of salyrganic acid, an organic mercurial which reacts with sulfhydryl groups,[7] completely abolishes the band. (3) Addition of one mole of silver nitrate removes the 375 mμ band almost completely. In view of the fact that serum albumin has been shown to have slightly less than one sulfhydryl group[1-3] per protein molecule, and since this –SH group reacts with mercurials as well as with silver ion before other side chains do,[2,3] it is clear that the disappearance of the copper absorption band at 375 mμ must be due to displacement of cupric ion from a mercaptide linkage.

With the establishment of the copper–sulfhydryl linkage, the 375 mμ band can be used as an indicator for the detection of other protein mercaptides. By means of this method, it has been found that one mole of Zn^{++} per mole albumin produces a detectable displacement of Cu^{++} from its sulfhydryl linkage, 10 moles of Zn^{++} a reduction of approximately 30% in the absorption at 375 mμ (Fig. 1) and 100 moles of Zn^{++} a reduction of about 80%. Even 100 moles of Zn^{++}, however, causes a drop of only a few per cent. in the intensity of the copper–albumin absorption near 700 mμ.[8] Thus Zn^{++} is not very effective in displacing Cu^{++} from side chains on the protein other than –SH.

Similar experiments have been carried out with Cd^{++} and with Pb^{++} as competing ions (Fig. 1). These indicate that the order of affinity for the sulfhydryl group of bovine albumin is: $Pb^{++} > Cd^{++} > Zn^{++}$.

Substantial formation of the zinc mercaptide occurs at a total Zn^{++} concentration as low as 0.003 *M*, even when this cation is competing with copper at an equivalent concentration. Interactions of zinc with the sulfhydryl group of albumin would not be distinguishable in equilibrium–dialysis experiments from binding by other side chains. Binding constants calculated on the assumption that only specified residues of the protein are active must take into account the preferential formation of the metal mercaptide.

These investigations were assisted by grants from the Office of Naval Research (Project No. NR124-054) and from the Carnation Company.

(7) The use of this compound was suggested by Dr. R. Benesch.

(8) I. M. Klotz and H. A. Fiess, *J. Phys. Colloid Chem.*, **55**, 101 (1951).

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Amino Acid Contamination in Preparations of Hog Blood Group Substances[1]

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In a previous communication,[2] some observations on the amino acid content of the dialyzable portion of mild acid hydrolysates of blood group substances were reported. Application of the chromatographic procedures of Sanger[3a,b] and Blackburn[4] to the dinitrophenyl (DNP) derivatives of the amino acids in the dialysate showed the presence of 0.07 to 0.7% free amino acids consisting of aspartic acid, glutamic acid, lysine, serine, threonine and glycine which were thought to represent a portion of the blood group substance proper. More recent studies have shown the necessity for re-evaluating this work.

In an effort to determine the dependence of the previously reported amino acid liberation on *p*H and time of hydrolysis, portions of a hog blood group substance were heated at *p*H 1.5, 1.9 and 2.3 for various periods of time. The free aspartic acid in the dialysate was found to reach a maximum value of around 0.2 mg. per 100 mg. of blood group substance for all three *p*H's. However, almost 0.1 mg. of aspartic acid per 100 mg. of the blood group substance was found in the control experiments in which the materials were dialyzed directly without being subjected to acid *p*H or heating, thus indicating the presence of substantial amounts of amino acid impurities. This was further borne out in the behavior of the other five amino acids which also appeared in significant amounts in the control dialysates.

A reinvestigation of several hog materials used in previous work[2] showed that they too were contaminated to the extent of about 0.4% with the same six free amino acids.

Thus all previous results are subject to considerable error depending on the extent of contamination of the particular substance with free amino acids. Moreover, the bulk of the 22–25% of amino acids known[5,6] to be present in the blood group substances is not liberated by such mild hydrolysis although the blood group activity is destroyed. More work will be necessary before the role of the amino acids in the structure of the blood group substances can be elucidated.

(1) Aided by grants from the United States Public Health Service and the William J. Matheson Commission.

(2) H. Van Vunakis and E. A. Kabat, THIS JOURNAL, **73**, 2977 (1951).

(3) (a) F. Sanger, *Biochem. J.*, **39**, 507 (1945); (b) R. R. Porter and F. Sanger, *ibid.*, **42**, 287 (1948).

(4) S. Blackburn, *ibid.*, **45**, 579 (1949).

(5) K. Landsteiner and R. A. Harte, *J. Expll. Med.*, **71**, 551 (1940).

(6) W. T. J. Morgan and H. K. King, *Biochem. J.*, **37**, 640 (1943).

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Mannich Reactions Involving the Use of Acetaldehyde, Monochloroacetaldehyde and Dichloroacetaldehyde[1]

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Mannich and co-workers[3] have described the condensation product obtained from dimethylamine hydrochloride, formaldehyde and acetaldehyde. They showed that an aldol condensation had ac-

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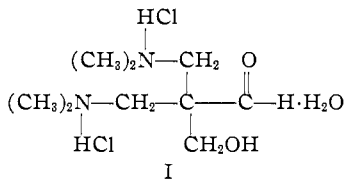
(2) This article is based on a thesis submitted by William D. Schaeffer in partial fulfillment of the requirements for the degree of Master of Science at Oregon State College, June, 1952.

(3) C. Mannich, B. Lesser and F. Silten, *Ber.*, **65**, 378 (1932).

companied the Mannich condensation. Our preliminary work confirmed this conclusion.



I

We have studied the Mannich condensation of monochloro- and dichloroacetaldehyde in an effort to determine the type of condensation which predominates as the number of available hydrogen atoms on the α-carbon atom of the aldehyde is reduced.

Monochloroacetaldehyde reacts according to the equation

$$ClCH_2CHO + 2CH_2O + (CH_3)_2NH\cdot HCl \longrightarrow (CH_3)_2NCH_2CCl(CH_2OH)CHO\cdot HCl$$

II

The isomeric structure $(CH_3)_2NCH_2CHClCH(OH)CHO\cdot HCl$ was eliminated as a possibility by the failure of the product to undergo oxidation to an α-keto acid.

The dichloroacetaldehyde also reacted with two moles of formaldehyde to produce the α-hydroxyaldehyde $(CH_3)_2NCH_2CCl_2CH(OH)CHO\cdot HCl$ (III), which formed a 2,4-dinitrophenylhydrazone and yielded upon oxidation an α-keto acid. Qualitative evidence of the formation of a 1,2-glycol was observed after reduction of the ketoaldehyde with sodium amalgam.

From the products of the reactions, no generalization can be made as to the type of condensation favored by the number and degree of reactivity of the hydrogen atoms on the α-carbon atom. However, by the use of Hirschfelder models it was found that in those cases in which an aldol condensation had taken place, the introduction of a dimethylaminomethyl group in its place would give rise to steric hindrance.

In the case of dichloroacetaldehyde the one replaceable hydrogen atom is free to undergo either the Mannich or aldol condensation, but yields upwards of 80% of the Mannich base are obtained. Therefore, the Mannich reaction must take precedence over the aldol condensation as long as spacial relationships are favorable.

Experimental

α-Chloro-α-(dimethylaminomethyl)-β-hydroxypropionaldehyde Hydrochloride.—Monochloroacetaldehyde, prepared from its acetal (30.5 g.) by heating with anhydrous oxalic acid, was distilled (b.p. 85–87°) directly into a flask containing 16.8 g. (0.206 mole) of dimethylamine hydrochloride, 6.3 g. (0.21 mole) of paraformaldehyde and 20 ml. of acid. The reactants were heated under reflux for 1 to 1.5 hours. When a pale yellow color developed, the solution was cooled in an ice-bath. Crystals were formed in the cold solution but final crystallization was effected by adding 100 ml. of boiling ethyl acetate. The hygroscopic compound was purified by dissolving in warm methanol and reprecipitating by the addition of acetone. The yield of product melting at 122–124° was 31%. Qualitative tests for carbonyl, primary alcohol and amine were positive.

Anal. Calcd. for $C_6H_{13}NO_2Cl_2$: neut. equiv., 202.06; C, 35.66; H, 6.48; N, 6.96; total Cl, 35.10; ionizable Cl, 17.55. Found: neut. equiv., 202.1; C, 35.48; H, 6.48; N, 7.02; total Cl, 35.19; ionizable Cl, 17.51.

The free base was prepared by the same method as described above. The long colorless crystals of the base were hygroscopic and unstable; decomposition was complete within a few hours. No quantitative data were collected. Qualitative tests for functional groups were positive.

α-Hydroxy-β,β-dichloro-β-(dimethylaminomethyl)-propionaldehyde Hydrochloride.—Seventeen grams (0.15 mole) of dichloroacetaldehyde, 12.7 g. (0.156 mole) of dimethylamine hydrochloride and 9.3 g. (0.31 mole) of paraformaldehyde were dissolved in 25 ml. of glacial acetic acid. The solution was heated under reflux for 1 to 1.5 hours until a pale yellow color developed. Crystals formed when the solution was cooled; final crystallization was effected by the addition of 50 ml. of boiling ethyl acetate. The crude product was recrystallized from boiling acetic acid. Yields of pure product m.p. 178° dec., ranged from 72–80%. Tests for carbonyl, primary alcohol and amine were positive.

Anal. Calcd. for $C_6H_{12}NCl_3O_2$: neut. equiv., 236.5; C, 30.47; H, 5.11; N, 5.92; total Cl, 44.97; ionizable Cl, 14.99. Found: neut. equiv., 236.2; C, 30.40; H, 5.17; N, 5.98; total Cl, 45.11; ionizable Cl, 15.10.

The free base was prepared by neutralizing with 3 *N* sodium hydroxide 3 g. of the hydrochloride dissolved in 5 ml. of water. The neutralized solution was cooled in an ice-bath. Colorless cubic crystals of the base were formed. The compound m.p. 63° was found to be unstable after long standing but the crystals were not hygroscopic. It appears by analysis to be a monohydrate. Tests for the carbonyl group, primary alcohol and amine were positive.

Anal. Calcd. for $C_6H_{13}NCl_2O_3$: C, 33.04; H, 6.07. Found: C, 32.60; H, 5.90.

Oxidation of the Hydrochloride to α-Keto Acid.—One-half gram of the hydrochloride dissolved in 5 ml. of distilled water was treated with an excess of a saturated solution of potassium permanganate. The mixture was stirred and cooled until a reaction was complete. The solution was cleared by the addition of sodium bisulfite. The solution was made acid with hydrochloric acid, and 2,4-dinitrophenylhydrazine reagent added. The crystals of the derivative were removed and recrystallized from 95% alcohol, m.p. 204°.

Anal. Calcd. for $C_{12}H_{14}N_5O_6Cl_3$: C, 33.5; H, 3.28. Found: C, 33.70; H, 3.26.

Reduction of the Hydrochloride to 1,2-Glycol.—To 3 g. of the hydrochloride dissolved in 20 ml. of distilled water, containing 0.5 ml. of concd. hydrochloric acid, was added with vigorous stirring, 15 g. of 2% sodium amalgam. The solution was neutralized with dilute sodium hydroxide and evaporated to dryness under reduced pressure. The residue was extracted with chloroform. Long colorless needles were formed as the chloroform evaporated. The reduced product, m.p. 120° with sublimation, was stable at room temperature and non-hygroscopic. Tests made on the compound for carbonyl were negative, and the Denige test[4] proved the absence of formaldehyde. Crystals of the compound were placed in a dilute solution of periodic acid and allowed to stand for 12 hours. A positive test for formaldehyde was then obtained by the Denige method.

(4) J. Walker, "Formaldehyde," Reinhold Publishing Corp., New York, N. Y., 1944, p. 244.

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Calculated Approximate Values of the Free Energy Function for the OD Molecule

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The values for the free energy function of the OD molecule as a harmonic oscillator have been calculated for the temperature range from 1000 to 3000°K. The values do not include the contribution due to nuclear spin. The values for the function are listed in Table I.