Racemic (*dl*)-hydrocarbon prepared in the same way had b.p. 168–170°, $n^{25}D$ 1.4130, m.p. −26.5°, and the same infrared spectrum.

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The Stability of N-Ethylmaleimide and its Reaction with Sulfhydryl Groups

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N-Ethylmaleimide (NEM) has been shown to react rapidly and specifically with sulfhydryl groups,[1] to be useful in the titration of such groups in proteins,[2] and to be an antimitotic agent.[1] In view of its potential application to the study of biochemical reactions involving sulfhydryl groups, we have made some observations on the rate of reaction and the stability of NEM.

Changes in the absorption spectrum of NEM on reaction with glutathione (GSH), in the region of 205–240 mμ, have been observed by Friedmann.[3] He shows also the broad peak centered at 302 mμ (molar extinction coefficient, ϵ_M 620), but does not mention its behavior in this reaction. The virtually complete disappearance of this peak, which takes place on combination with mercaptans, or on decomposition of NEM, has been used here to follow the rate of reaction and the instability of the compound at various *p*H values.

For 1.25×10^{-3} *M* solutions of NEM in water or 0.05 *M* buffers, the change in optical density with time at 302 mμ is shown in Fig. 1, I. The first-order reaction taking place at *p*H values above neutrality is probably a hydrolysis of the imide linkage since the resulting product combines with mercaptans only very slowly and has an absorption spectrum indistinguishable from that of synthetic N-ethylmaleamic acid. This instability should be taken into account in any quantitative procedure using NEM, such as the titration of sulfhydryl groups,[2] in which there is any possibility of the reagent being exposed to alkaline conditions.

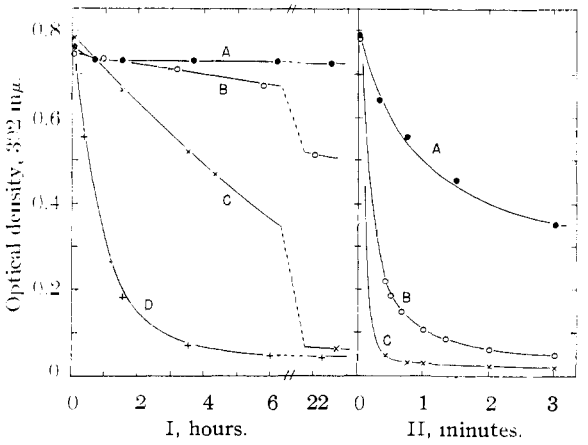

Fig. 1.—I, rate of decomposition of 1.25×10^{-3} *M* NEM in water or 0.05 *M* potassium acetate, *p*H 5.0 (A); potassium phosphate, *p*H 7.0 (B); tris-(hydroxymethyl)-aminomethane, *p*H 8.0 (C); 2-amino-2-methyl-1,3-propanediol, *p*H 9.0 (D). II, rate of reaction of NEM with GSH (both 10^{-3} *M*) in 0.1 *M* sodium acetate, *p*H 5.0 (A); potassium phosphate, *p*H 6.1 (B), and *p*H 7.0 (C).

The rate of reaction of NEM with GSH as a function of *p*H is shown in Fig. 1, II. Clearly, at about neutrality, the reaction with GSH is so much faster than the decomposition of the reagent that it may be used for quantitative purposes. The absolute change in molar extinction coefficient ($\Delta\epsilon_M$ 0.61 × 10^3), however, is only one-twelfth of that observed by Boyer[4] at 250 mμ when *p*-mercuribenzoate reacts with mercaptans, ($\Delta\epsilon_M$ 7.6 × 10^3), and in general it is probably not sensitive enough for photometric titration of protein sulfhydryl groups.

On the basis of this optical method, there is no evidence of appreciable reaction of NEM at *p*H 7 with S-acetyl GSH, diacetyl 2-mercaptoethylamine, oxidized glutathione, ethanol, ethylamine or HCN. Cysteine and H_2S react rapidly, as does potassium borohydride at *p*H 9. For comparison, some determinations have been made of the rate of combination of NEM with myokinase as assessed by inactivation of the enzyme. At *p*H 7.5 maximal inactivation (95%) was not reached until after treatment for 30 minutes with 2×10^{-4} *M* NEM. Excess NEM must have been present, since at *p*H 9 as little as 5×10^{-5} *M* NEM gave the same inactivation in 5 minutes. The time curve of inactivation was smooth and showed no evidence for classes of sulfhydryl groups having different reactivities.[5]

The situation with this enzyme is not clear, however, since the original preparation was activated fivefold when assayed in the presence of GSH; yet in the original state it was susceptible to almost complete inactivation by NEM. The GSH activation is thus not explainable simply by the existence of sulhydryl groups as disulfides, in which condition they should be immune to the inhibitor.

Experimental

Spectra were obtained in a Cary Recording Spectrophotometer, and reactions were followed in a Beckman Model DU Spectrophotometer.

N-Ethylmaleamic acid was synthesized from maleic anhydride and ethylamine as described by Piutti.[6]

Myokinase was prepared from horse muscle, essentially by the procedure of Colowick and Kalckar[7] with the addition of a fractionation between 17.5 and 35% saturated ammonium sulfate. The enzyme was assayed in a system containing 200 μ*M* tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.5, 5 μ*M* magnesium chloride, 2.5 μ*M* adenosine diphosphate, 20 μ*M* GSH, 30 μ*M* glucose, and excess hexokinase in 1.0 ml., by measuring loss of acid-labile phosphate. Of the above preparation, 3 μg. of protein transferred 1.0

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μM phosphate in 10 minutes at 30°. Inactivation was carried out by incubating solutions containing 0.25 mg. per ml. protein and 2×10^{-4} M NEM at room temperature, and diluting with a large excess of GSH at appropriate time intervals.

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Laboratory Preparation of Tetrakis-(hydroxymethyl)-phosphonium Chloride

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Tetrakis-(hydroxymethyl)-phosphonium chloride, $(HOCH_2)_4PCl$, which is made by treating phosphine with an aqueous solution of formaldehyde–hydrochloric acid, recently has attracted interest as an ingredient of certain resins which impart flame-resistance to cotton fabrics.[2a,b] The only available method for making this compound was reported by Hoffman in 1921.[3] His method is slow and somewhat hazardous, since frequent minor explosions occur during the reaction. Since it appeared that tetrakis-(hydroxymethyl)-phosphonium chloride, abbreviated THPC for convenience, would be in demand for laboratory and perhaps commercial use, improvements were required in the details of its preparation. The purpose of this report is to present a practical laboratory method for preparing THPC.

Heretofore, a temperature of 80° was considered most desirable and was used for treating phosphine with an aqueous solution of formaldehyde and hydrochloric acid.[3] At lower temperatures the reaction was thought to be slow and at higher temperatures the vapor pressure of the solution interfered with the absorption of phosphine. Since phosphine is spontaneously inflammable at 80°, frequent explosions occurred in the reaction vessel of the Hoffman method. By that method it required about 8 hours for the phosphine generated from 30 g. of aluminum phosphide to be absorbed and to react with 100 g. of formaldehyde–hydrochloric acid solution.

We have found that phosphine will react rapidly with formaldehyde–hydrochloric acid solutions at room temperature. Phosphine is more soluble in these solutions at 25° than at 80°, and at the lower temperature explosions are eliminated. If the reaction is carried out at 50°, explosions occur only occasionally. However, since heating is not necessary for the reaction, a temperature of about 25° is preferred; temperatures of 10 to 15° also are satisfactory. It appears that the primary factor which controls the amount of THPC that can be made in the laboratory from a specified amount of formaldehyde–hydrochloric acid solution in a given length of time is the amount of phosphine that can be made available. With two small laboratory generators, sufficient phosphine was made in 30 hours to make over 1100 g. of THPC. In this case the phosphine produced by 30 g. of aluminum phosphide was absorbed and allowed to react with 100 g. of formaldehyde–hydrochloric acid solution in less than one hour.

A good yield of THPC and an easily isolated product is obtained when about 4.2 moles of formaldehyde are used per mole of hydrochloric acid. Paraformaldehyde or polyoxymethylene may be substituted for the formaldehyde without affecting the rate of the reaction or yield of product.

The phosphine used may be made by any method desired. Relatively pure phosphine is obtained conveniently by treating aluminum phosphide with water at room temperature. This source supplies a steady stream of the gas. Aluminum phosphide is not generally available, but it can be made by igniting with adequate precautions a mixture of 1.5 to 2.0 parts (by weight) of powdered aluminum and one part of red phosphorus.

Experimental

A. Apparatus.—The apparatus consisted of two phosphine generators provided with safety water-seal pressure releases, and a reaction vessel. Each generator consisted of a two-liter filter flask fitted with a two-hole rubber stopper. Through one of the holes was an inlet tube used to admit nitrogen and the other was connected to a safety water-seal pressure release. The water-seal pressure release could be of any convenient design as long as the water levels in it can be varied from 0 to about 15 inches. The side arm of each filter flask was connected to the reaction vessel. The reaction vessel was a five-liter jar fitted with a four-hole wooden cover. The inlet tubes, which consisted of gas dispersion filter tubes, passed through two of the holes and extended nearly to the bottom of the vessel. A high speed stirrer and a vent tube were mounted through the other two holes.

B. Reagents.—(1) Nitrogen. (2) Formaldehyde–hydrochloric acid solution was prepared by mixing 37% formaldehyde and 35% hydrochloric acid so that the final solution contained these reagents in about a 4.2:1 mole ratio, respectively. (3) Aluminum phosphide: about 100-g. portions of a mixture of 528 g. of powdered aluminum and 352 g. of red phosphorus were placed on an asbestos mat in a fume hood and ignited with a match. The combined portions made about 880 g. of crude aluminum phosphide. Small portions of the aluminum–phosphorus mixture were ignited because intense heat and toxic vapors are produced when these elements are burned. (4) Phosphine was generated by adding aluminum phosphide to water in the apparatus described above.

C. Procedure.—All operations were performed in a well ventilated hood because of the toxicity of phosphine. About 1600 ml. of water was added to each phosphine generator, then water was added to each safety water-seal pressure release tube. Formaldehyde–hydrochloric acid solution, made by combining 660 g. of 35% aqueous hydrochloric acid and 2100 g. of 37% aqueous formaldehyde, was put into the reaction vessel. The stirrer in the reaction vessel was adjusted for vigorous agitation and then the apparatus was swept out with nitrogen by allowing a steady stream of the nitrogen to pass through it for about 10 minutes. At the time the nitrogen was stopped, about 5 g. of aluminum phosphide was added to each generator. After about one hour, 15 g. of aluminum phosphide was added to each generator and so on at hourly intervals until the 880 g. had been added. Two hours after the last addition of aluminum phosphide the reaction vessel was disconnected and the solution was transferred to large evaporating dishes. The volatile components were evaporated at about 70 to 75° with stirring until crystals began to form, when the mass was transferred to a desiccator and allowed to cool to room temperature over sodium hydroxide pellets. The entire mass crystallized. Approximately 1135 g. of 95% THPC

(1) Deceased.

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