Spectrophotometric Assay for Reaction of N-Ethylmaleimide with Sulfhydryl Groups

ARTICLE *in* ANALYTICAL CHEMISTRY · JULY 1958

Impact Factor: 5.64 · DOI: 10.1021/ac60139a030

No. 52

"the hydrolysis is more rapid in an alkaline solution than in an acid solution of the same strength." In a solution of pH 6, in which the initial cadmium ion and the thioacetamide concentrations are 0.010 and 0.10*VF*, respectively, the time required for half of the cadmium to precipitate by the direct reaction is less than 9 minutes at 90° C. In this time and under these conditions, the cadmium sulfide precipitated by the hydrolysis-controlled reaction would be less than 10^{-6} mole. This indicates that the more rapid precipitation observed at higher pH values could be caused by the direct reaction mechanism rather than an increase in the rate of hydrolysis. An investigation of the hydrolysis of thioacetamide in alkaline solutions is in progress.

The velocity constant (*4*) for the precipitation of lead sulfide by thioacetamide in the pH range 5.1 to 3.5 was 1.15×10^{-3} liter$^{1/2}$ mole$^{-1/2}$ minute^{-1}. This is 42% higher than the constant for the precipitation of cadmium sulfide in the same pH range. The concentration of cadmium(II) in a saturated solution at a given sulfide concentration should be 52% greater than that of lead(II) in a similar solution (*3*). There may be some correlation between this increase in solubility and the decrease in the velocity constant. Such a relationship cannot be postulated until the velocity constants for the rate of precipitation of other basic cations are determined.

ACKNOWLEDGMENT

Preliminary experiments by L. B. Marantz were of value in planning this investigation. Comments on the manuscript by R. C. Greenough have been helpful.

LITERATURE CITED

(1) Barber, H. H., Taylor, T. I., "Semimicro Qualitative Analysis," Harper, New York, 1953.
(2) Butler, E. A., Swift, E. H., ANAL. CHEM. 29, 419 (1957).
(3) Kolthoff, I. M., *J. Phys. Chem.* 35, 2711 (1931).
(4) Swift, E. H., Butler, E. A., ANAL. CHEM. 28, 146 (1956).

RECEIVED for review August 5, 1957. Accepted January 30, 1958. Contribution No. 2181 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, Calif. Supported by the National Science Foundation.

Spectrophotometric Assay for Reaction of *N*-Ethylmaleimide with Sulfhydryl Groups

EUGENE ROBERTS and GEORGE ROUSER

Department of Biochemistry, City of Hope Medical Center, Duarte, Calif.

▶ **Ultraviolet spectral changes accompanying the interaction with *N*-ethylmaleimide in dilute aqueous solution at pH 6.0 can be utilized for the quantitative estimation of sulfhydryl compounds. The maximal changes in absorbance occur at wave lengths at which minimal interference would occur from many ultraviolet-absorbing substances of biological interest.**

SINCE the first report of the quantitative reaction of *N*-ethylmaleimide with thiol groups (*5*), this type of reaction has been applied to the determination of various sulfhydryl compounds on paper chromatograms (*1*, *8*) and in tissue sections (*9*). Changes in the ultraviolet absorption spectrum of *N*-ethylmaleimide upon reaction with sulfhydryl compounds have been observed (*4*, *6*), and it has been suggested that these changes could be used for quantitative purposes (*6*).

The present report shows that at pH 6.0 the loss in absorption of *N*-ethylmaleimide at 300 mμ, the absorption maximum, can be used for the quantitative estimation of sulfhydryl groups in dilute solution in the presence of excess *N*-ethylmaleimide. Thus, 1 μmole of cysteine per ml. yields an absorbance change of 0.60. The change in absorbance is proportional to the concentration of cysteine in the range studied. Interference by a number of compounds of biochemical interest, such as purines, pyrimidines, nucleosides, and nucleotides, is minimal at the wave length and pH employed. If necessary, wave lengths up to 320 mμ can be used without too great a loss in sensitivity. This procedure overcomes the objections to the spectrophotometric method for following the reaction of *p*-chloromercuribenzoate with sulfhydryl groups at 250 mμ (*2*) and yet makes possible kinetic measurements without removal of individual samples for analysis (*3*) from a reaction mixture.

EXPERIMENTAL

Reagents. L-Cysteine hydrochloride monohydrate and glutathione were purchased from Schwarz Laboratories, Inc., and crystallized bovine plasma albumin from Armour and Co. The *N*-ethylmaleimide was obtained from Delta Chemical Works, Inc. Buffers were prepared from analytical reagent grade salts.

Apparatus. All determinations were made with a Beckman Model DK-2 recording spectrophotometer. Kinetic measurements were made with the aid of an adapter which allows continuous measurement at a given wave length, the curve progressing at the rate of 1 inch per minute.

RESULTS

Spectral shifts accompanying reaction of cysteine with *N*-ethylmaleimide are shown in representative curves (Figure 1). The spectrum of *N*-ethylmaleimide, curve 1, has a maximum at 300 mμ and a minimum at 248 mμ. Cysteine alone has no distinctive spec-

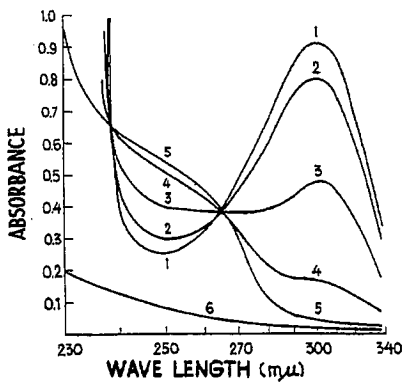

Figure 1. Absorption spectra

0.1 *M* phosphate buffer, pH 6.0

1. 1.5×10^{-3} *M* *N*-ethylmaleimide
2. 0.15×10^{-3} *M* cysteine with 1.5×10^{-3} *M* *N*-ethylmaleimide
3. 0.75×10^{-3} *M* cysteine with 1.5×10^{-3} *M* *N*-ethylmaleimide
4. 1.27×10^{-3} *M* cysteine with 1.5×10^{-5} *M* *N*-ethylmaleimide
5. 1.50×10^{-3} *M* cysteine with 1.5×10^{-3} *M* *N*-ethylmaleimide
6. 1.5×10^{-3} *M* cysteine

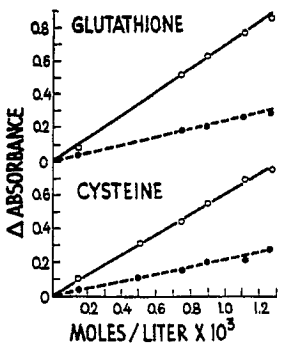

Figure 2. Changes in absorbance of 1.5 × 10⁻³M N-ethylmaleimide as function of glutathione or cysteine concentration

0.1M phosphate buffer, pH 6.0
——— Decrease at 300 mμ
····· Increase at 248 mμ

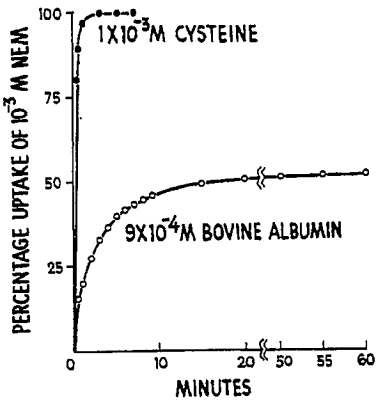

Figure 3. Rates of reaction of cysteine and bovine plasma albumin with N-ethylmaleimide at 25° C.

Points plotted from continuous curves of spectrophotometric record

tral characteristics at these wave lengths (curve 6). Solutions of 1.5 × $10^{-3}M$ N-ethylmaleimide with increasing concentrations of cysteine up to 1.5 × $10^{-3}M$ produced progressive decreases in absorbance at wave lengths between 265 and 340 mμ and increases between 238 and 265 mμ (curves 2 to 5 are typical). There was an isomolar reaction between cysteine and N-ethylmaleimide. Similar results were obtained with glutathione. The results in Figure 2 show the changes in absorbance at 300 and 248 mμ as a function of the concentration of cysteine or glutathione. Linearity of response was obtained over the range tested, with relative change greatest at 300 mμ. Similar plots could be made for the changes in absorbance at other wave lengths. The slightly greater changes in absorbance obtained for glutathione than for cysteine at comparable molarities are probably due to small amounts of sulfhydryl impurities in the sample of glutathione used.

Results of kinetic experiments in which reaction rates of N-ethylmaleimide with cysteine and bovine plasma albumin were measured are shown in Figure 3. The stoichiometric interaction of cysteine and N-ethylmaleimide was complete in 2 minutes after mixing, agreeing with a previous report (5). The reaction with bovine albumin was much slower, a final value being obtained at 55 minutes which corresponded to the presence of 0.60 sulfhydryl groups per albumin molecule. This is comparable to the value of 0.71 obtained by amperometric titration (7) but lower than the value of 1.0 shown by mercurimetric determination (3). The reaction of N-ethylmaleimide with myokinase, as judged by inactivation of the enzyme, also took place slowly at pH 7.5 (6).

Variations of the method described should prove useful in a variety of biochemical studies.

LITERATURE CITED

(1) Benesch, Reinhold, Benesch, R. E., Gutcho, Marcia, Laufer, Louis, *Science* 123, 981–2 (1956).
(2) Boyer, P. D., *J. Am. Chem. Soc.* 76, 4331–7 (1954).
(3) Fridovich, Irwin, Handler, Philip, ANAL. CHEM. 29, 1219–20 (1957).
(4) Friedmann, E., *Biochim. et Biophys. Acta* 9, 65–75 (1952).
(5) Friedmann, E., Marrian, D. H., Simon-Ruess, I., *Brit. J. Pharmacol.* 4, 105–8 (1949).
(6) Gregory, J. D., *J. Am. Chem. Soc.* 77, 3922–3 (1955).
(7) Jensen, E. V., Hospelhorn, V. D., Tapley, D. F., Huggins, Charles, *J. Biol. Chem.* 185, 411–22 (1950).
(8) Price, C. A., Campbell, C. W., *Biochem. J.* 65, 512–16 (1957).
(9) Tsou, Kwan-Chung, Barrnett, R. J., Seligman, A. M., *J. Am. Chem. Soc.* 77, 4613–16 (1955).

RECEIVED for review September 13, 1957. Accepted February 25, 1958. Investigation supported by research grants C-2568 and C-3134 from the National Cancer Institute, U. S. Public Health Service.

Spectrophotometric Assay for Sulfhydryl Groups Using N-Ethylmaleimide

NICHOLAS M. ALEXANDER

Radioisotope Service, Veterans Administration Hospital, West Haven, Conn., and the Department of Biochemistry, Yale University, New Haven, Conn.

▶ A rapid, simple spectrophotometric method for determining thiols with N-ethylmaleimide is presented. The reaction is observed at 300 mμ. Sulfhydryl solutions having concentrations as low as 0.0001M can be assayed. The procedure has been successfully used to determine sulfhydryl concentrations in a tissue extract and in whole blood.

N-ETHYLMALEIMIDE (NEM) reacts rapidly with sulhydryl compounds at neutral pH (4, 5). The rate of reaction of equimolar amounts of N-ethylmaleimide and reduced glutathione has been followed spectrophotometrically by a decrease in absorption of the former at 300 mμ (5), but the reaction does not go to completion under these conditions. Roberts and Rouser (7) showed that the change in absorbance at 300 mμ is proportional to the concentrations of cysteine and glutathione when N-ethylmaleimide is present in excess. They used this method to determine the extent to which bovine serum albumin reacts with N-ethylmaleimide.

The present report also shows that, when present in excess, N-ethylmaleimide reacts stoichiometrically with sulfhydryl compounds. The decrease in absorption of this compound at 300 mμ can be used as an assay method for sulfhydryl groups.

EXPERIMENTAL

Materials. Reduced glutathione (GSH) and N-ethylmaleimide were obtained from Schwarz Laboratories, Inc., Mount Vernon, N. Y. Cysteine