and the predictions of the dipole interaction theory, if the changes are no larger in the case of these solutes.

Although the agreement is somewhat better than in the case of the slopes, the experimental values are still two or three times as great as the theoretical predictions.

Acknowledgment.—It is a pleasure to acknowledge the assistance of grants-in-aid from the Abbott Fund of Northwestern University which made this work possible, and to Chester T. O'Konski in developing the electronic circuits.

Summary

We have described an ultrasonic interferometer designed for the precise measurement of wave lengths in aqueous solutions at a frequency of about 4 megacycles. With the final design of the apparatus, successive experiments agreed to about 0.02%, and the average deviation of measurements on a series of solutions of different concentrations was 0.05% from a smooth curve.

We have measured a series of aqueous solutions of glycine at 25°, and calculated the adiabatic compressibility and apparent molal compressibilities, which we find to be linear functions of the concentration in the very dilute solution. Preliminary results on glycolamide and on α- and β-alanine and lactamide show that these solutes also behave much like glycine.

The Fuoss–Kirkwood theory of dipolar interaction and the Kirkwood theory of the energy of solvation of a dipolar solute give qualitative although not quantitative agreement with our experimental results.

EVANSTON, ILLINOIS RECEIVED AUGUST 8, 1949

[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY]

Inhibition of Urease by Sulfur Compounds

BY JOHN F. AMBROSE,* G. B. KISTIAKOWSKY† AND ANDREW G. KRIDL‡

In a recent investigation Kistiakowsky and Lumry[1] found that the anomalous temperature effects in the hydrolysis of urea by urease in the presence of sulfite ions can be explained by inhibition of the enzyme by these anions. This inhibition, heretofore unreported in the literature, seemed to merit further investigation, all the more so since sulfite has been used as a "preservative" for urease solutions.

The purpose of the investigation was to elucidate the laws of inhibition with respect to the sulfite ions, temperature and *p*H dependence of the equilibrium constant for the inhibition reaction, to determine the type of inhibition caused by the anions, and to see whether organic derivatives of sulfur dioxide and/or sulfur trioxide also possess inhibitory action.

Experimental Details

The activity of the urease samples was determined by the Nesslerization technique of Sumner and Hand[2] with only minor modification. A Lumetron Colorimeter manufactured by the Photovolt Corporation was used for transmission measurements. The reproducibility with this instrument was excellent, the standard deviation being in all instances less than 1%.

Temperature control was provided by water-filled thermostats with liquid cooling coils, electric heating elements and automatic temperature controls such that the temperatures could be maintained to ±0.01° over the short periods of time (about ten minutes) necessary to perform individual experiments. Temperatures were read with Beckmann thermometers calibrated against a Bureau of Standards calibrated mercury thermometer.

An activity determination always consisted of pipetting 20 cc. of an urease–phosphate buffer–sulfite solution into an ordinary test-tube, placing the latter in the thermostat, waiting until temperature equilibrium has been established, and then adding, with a thirty-second stirring, 1 cc. of urea solution containing 315 g. of urea per liter. Upon dilution this gave a 1.5% solution of urea, under which conditions the reaction is of zero order in substrate. Following the mixing the reaction mixture was left in the thermostat for a total of five minutes after which time the reaction was stopped by rapid addition from a hypodermic syringe of 1 cc. of 7 *N* sulfuric acid followed by rapid mixing for about twenty seconds. Two cc. of this reaction mixture was then diluted to 250 cc. with ammonia-free water in a volumetric flask, and 5 cc. of Nessler reagent added. The transmission of the resulting solution was determined after a ten-minute waiting period.

Using 20-cc. volumes of reaction mixture was, to be sure, somewhat wasteful of reagents but assured much higher over-all reproducibility and precision than were obtained using the previous procedure.[1]

The urease solutions used in these experiments were identical with those previously described.[1] However, better preparative and storage procedures resulted in urease stock solutions which were so stable that the activities remained un-

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‡ Monsanto Predoctoral Fellow.

(1) G. B. Kistiakowsky and Rufus Lumry, THIS JOURNAL, **71**, 2006 (1949).

(2) J. B. Sumner and D. B. Hand, *J. Biol. Chem.*, **76**, 149 (1928).

changed over periods of several days. The effect described by Niemann, *et al.*,[3] that the activity of dilute urease solutions increases with standing was also observed by us.

For the determination of the effect of oxidation–reduction potential on the activity of urease, crystalline enzyme prepared by Worthington Chemical Company, Freehold, New Jersey, was used.

The inhibition was determined at different *p*H's. The buffers used in all cases were appropriate mixtures of potassium dihydrogen phosphate and disodium phosphate. Special buffer salts obtained from Fischer Scientific Company were used throughout without further purification. The sulfite solutions used were made up to the same *p*H as the phosphate buffer by using mixtures of sodium sulfite and sodium bisulfite of reagent grade. The sulfite solutions were always prepared fresh for each experiment to minimize complications due to the air oxidation.

Urea used was Eastman Kodak Co. technical product, the C. P. reagent having been shown to give identical results.

All the water used in these experiments was distilled once through a permanganate still and then once more through an all-glass apparatus.

The compositions of representative buffers and sulfite solutions were

*p*H	KH_2PO_4 g./l.	Na_2HPO_4 g./l.	$NaHSO_3$ g./l.	Na_2SO_3 g./l.
5.73 ± 0.03	85.6	9.94	86.3	21.4
6.93 ± .03	28.7	69.5	22.6	101.4
7.63 ± .03	9.55	168.1	5.21	119.8

In all cases the molarity of stock solutions of phosphate buffer was 0.700 and of sulfite solutions 1.02. The phosphate buffer was always diluted 1:1 so that its molarity in the reaction mixture was 0.350. Various concentrations of sulfite were used (see below).

The capacity of the buffers used was such that during a five-minute run the *p*H change was never more than 0.1–0.2 unit.

Other details of the procedure were identical with those previously described.

Solutions of C.P. sodium sulfate, sodium benzenesulfinate and sodium benzenesulfonate were used without further purification.

The procedure developed to determine whether inhibition was competitive or not was similar to that described recently by Niemann and Harmon.[4] Solutions of urea giving 5, 6, 7, 8, 10, 15, 25 and 50 millimoles/l. upon dilution with the reaction mixture were prepared by appropriately diluting a stock solution containing 63 g. of urea per liter. In these experiments reaction times shorter than five minutes were used, namely, either three or two minutes, and the dilutions for Nesslerization varied from 2 to 10 cc. to 100 cc. using then only 2 cc. of Nessler reagent. In this series of experiments 0.112 *M* phosphate buffer of *p*H 7.0 was used, its composition being 4.99 g. of potassium dihydrogen phosphate and 12.09 g. of dibasic sodium phosphate per liter of solution.

(3) J. Peterson, K. M. Harmon and C. Niemann, *J. Biol. Chem.*, **176**, 1 (1948).

(4) K. M. Harmon and C. Niemann, *ibid.*, **177**, 601 (1949).

Results

The procedure used to determine the order of inhibition was that of Eyring, *et al.*[5] Let A_0 be the uninhibited activity of the enzyme, A its activity in the presence of inhibitor and K the equilibrium constant for the reaction $E + n(SO_3^=) \rightleftarrows E(SO_3^=)_n$ (inactive) where E is an active site and $(SO_3^=)$ denotes the total sulfite concentration, that is $[SO_3^=] + [HSO_3] + (H_2SO_3)$, the last being negligible.

Then $\log [(A_0/A) - 1] = \log K + n \log [SO_3^=]$ (1) and plotting the left-hand side of (1) *vs.* $\log (SO_3^=)$ there is obtained a straight line whose slope equals n, the kinetic order with respect to the inhibitor.

Using this technique a *first order dependence* on sulfite concentration was obtained over the *p*H range from 5.7 to 7.4 and at three different temperatures 8.5, 15 and 25°. Some of the representative curves are shown in Fig. 1. The fractional inhibition of the enzyme was found to be independent of enzyme concentration.

It will be noted from equation (1) that when $A_0 = 2A$ the log $(SO_3^=)$ becomes equal to the negative of log K. This method of estimating $K_{eq.}$ is, however, not a very convenient one since the results depend very strongly upon the choice of a straight line through the experimental points

TABLE I

EQUILIBRIUM CONSTANTS FOR THE SULFITE INHIBITION

T, °C.	*p*H	$K_1 = \frac{(A_0 - A)}{A(SO_3^=)_{total}}$	$K_2 = \frac{(A_0 - A)}{A(HSO_3^-)}$ Both *K*'s are in l./mole
25	6.14	22	1.7×10^2
	6.50	11	1.8×10^2
	6.80	5.4	1.7×10^2
	7.05	2.6	1.5×10^2
	7.45	1.6	2.1×10^2
		Average	1.8×10^2
15	6.27	34	3.3×10^2
	6.52	20	3.4×10^2
	6.87	8.2	2.9×10^2
	7.16	4.5	3.1×10^2
	7.60	2.7	3.9×10^2
		Average	3.3×10^2
8.5	6.20	61	5.2×10^2
	6.50	29	4.7×10^2
	6.93	13	5.1×10^2
	7.12	6.6	3.9×10^2
	7.55	2.4	4.1×10^2
		Average	4.6×10^2

(5) Eyring, *et al.*, *J. Gen. Physiol.*, **28**, 463 (1944).

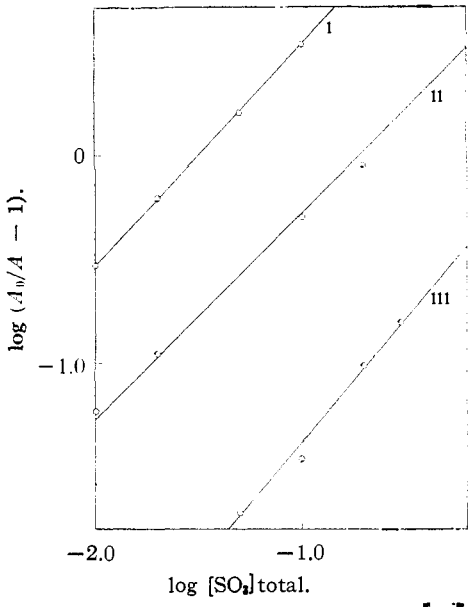

Fig. 1.—The order of inhibition in sulfite of urease solutions: line I, at *p*H 5.73, 25°; line II, *p*H 6.93, 8.5°; line III, *p*H 7.63, 25°.

and on the accuracy with which the intercept can be read. A more direct method of obtaining $K_{eq.}$ is to form an expression $K = (A_0 - A)/A(SO_3^=)$. It should be noted here that the concentrations of sulfite used in these experiments were of the order of 0.1 *M* and those of the urease of the order of 10^{-9} *M*, so that the use of the total sulfite concentration in the above constant is fully justified.

The summary of these constants will be found in Table I in the column K_I. It will be noted that these constants are strongly *p*H dependent, increasing quite rapidly with decreasing *p*H. In this *p*H range sulfite is present largely in the form of $SO_3^=$ ions whose concentration, therefore, is virtually independent of *p*H. Hence inhibition is not caused by this ion. The proportion of the HSO_3^- ion does, however, increase with the *p*H and the extent of inhibition was correlated with the concentration of the bisulfite ion using the value 5×10^{-6} for its ionization constant.[6] The results of this calculation are summarized under K_2. It will be noted that the variation with *p*H is moderate and fairly random. When the log of the average of these K_2's is plotted *vs.* $1/T$, a straight line results giving as the heat of inhibition 9,200 cal. per mole of active centers (Fig. 2).

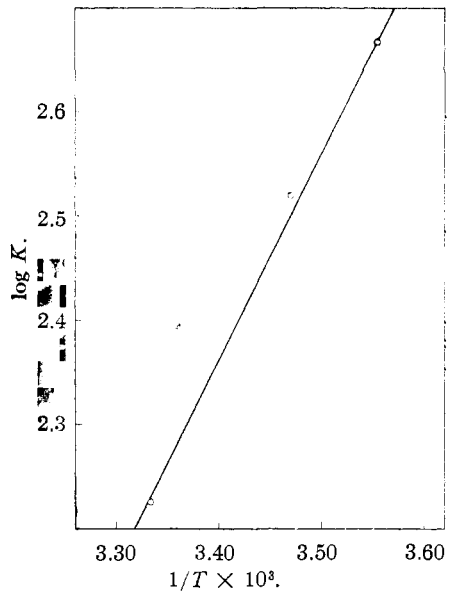

Fig. 2.—A plot of the logarithms of the sulfite inhibition constant against inverse temperature.

Sodium sulfate, sodium benzenesulfinate and sodium benzenesulfonate were also investigated. As shown in Fig. 3, at the same molar concentration the sulfinate is a stronger inhibitor than sulfite, although this order is inverted when inhibitions are calculated for equal concentrations of bisulfite and sulfinate ions. The sulfonate is a still weaker inhibitor, whereas the sulfate in-

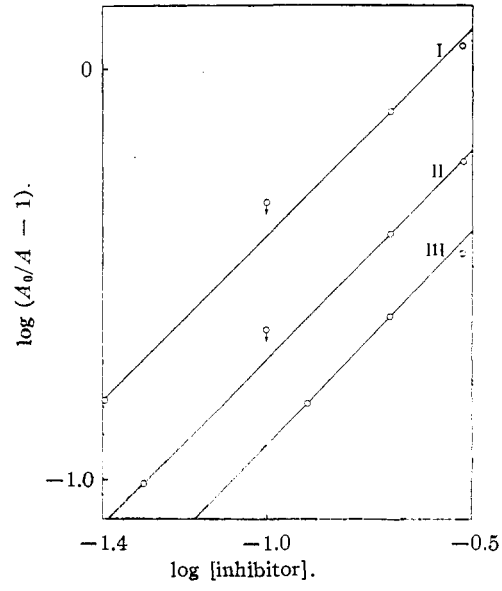

Fig. 3.—Comparative strength of inhibition at 25° and *p*H 6.93: line I, $C_6H_5SO_2Na$; line II, $NaHSO_3$ and Na_2SO_3; line III, $C_6H_5SO_3Na$.

(6) "International Critical Tables." A lower value 1×10^{-7} has been given by Latimer ("Oxidation Potentials," Prentice-Hall, Inc., New York, N. Y., 1938), whereas Lange's "Handbook of Chemistry," Sixth Edition selects the value of 6×10^{-8}. When use is made of one of these more recent values, the inhibition of urease cannot be attributed to bisulfite ions. A consistent representation of the experimental data is obtained on assuming that both the sulfite and bisulfite ions have the same inhibiting action and that the equilibrium constant is inversely proportional to the hydrogen ion concentration. The standard entropy change in this case is zero. As these findings are less rational than those derived from a higher value of the ionization constant, we chose to use the latter. We are indebted to Dr. T. R. Hogness for calling our attention to the higher ionization constant.

hibits very little if at all. The inhibitions are all of first order in the inhibitors.

The inhibition by the phenyl sulfinate ions was found to be essentially independent of the *p*H (Table II) after care was taken to prevent air oxidation of sulfinate in the course of making and using its solutions.

TABLE II

EQUILIBRIUM CONSTANTS FOR PHENYL SULFINATE INHIBITION AT 25°

*p*H	$K_I = \frac{(A_0 - A)}{A(\phi SO_2^-)}$
6.01	4.35
6.61	5.02
6.99	4.06
7.05	4.09

Qualitative experiments on sodium benzenesulfonate showed some dependence on *p*H.

Since Harmon and Niemann[4] have shown that the phosphate ion is a competitive inhibitor the following equation must be applied to test the competitive nature of the inhibition by another species, present simultaneously and assumed to act independently

$$\frac{1}{V} = \frac{K_s}{V_{max.}}\left(1 + \frac{(I_1)}{K_1} + \frac{(I_2)}{K_2}\right)\frac{1}{S} + \frac{1}{V_{max.}}$$

where

V = reaction velocity
K_s = dissociation constant for the enzyme–substrate complex
K_1 = dissociation constant for the enzyme–inhibitor 1 complex
K_2 = dissociation constant for the enzyme–inhibitor 2 complex
(I_1) = concentration of the inhibitor 1 (phosphate)
(I_2) = concentration of the inhibitor 2 (sulfite)
(S) = concentration of the substrate
$V_{max.}$ = maximum velocity (at urea concn. ~1.5%)

Thus plotting $1/V$ *vs.* $1/S$ there should be obtained a family of straight lines, and if the inhibition is competitive, *i. e.*, if substrate and the two inhibitors all compete for the same active site, only the slope should change, the intercept, $1/V_{max.}$, remaining constant. Such is indeed the case as shown by Fig. 4.

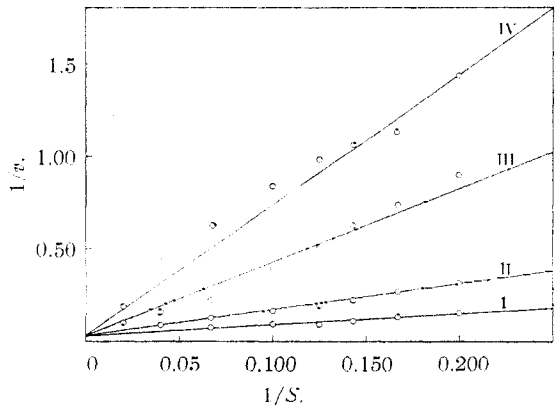

Fig. 4.—The competitive nature of the inhibition by sulfite in 0.056 *M* phosphate buffers: line I, no sulfite; line II, 0.040 *M* sulfite; line III, 0.100 *M* sulfite; line IV, 0.160 *M* sulfite.

It has been claimed by Sizer[7] that the activity of crystalline urease is a function of the oxidation–reduction potential of the solution, the crude extract being, however, unaffected by it.

Since a crude extract has been used throughout these experiments, it was decided to try to ascertain in a qualitative way whether crystalline enzyme has any such dependence.

The method used was to hold the sulfite concentration constant and vary the sulfate, and then hold the sulfate constant and vary the sulfite, thus changing the oxidation–reduction potential of the solution.

In both cases the data could be accounted for on the basis of the cumulative inhibitory effect of sulfite and sulfate, the oxidation–reduction effect of this couple being undetectable.

Technical difficulties prevented a similar test of the oxidation–reduction couples formed by sulfite and lower oxidation states of sulfur, *i. e.*, thiosulfate, etc.

Discussion

The present study of the inhibition of urease activity has not progressed far enough to permit definite conclusions as to the nature of the inhibition reaction or, what is really far more important, of the nature of the active sites of urease. Some comments are, however, in order. From the observed reaction heat and the values of the equilibrium constants, the standard entropy change, with moles per liter as standard states, is readily computed as −21 e.u. This change when compared with those observed for various other reactions in aqueous medium, suggests that the process involved is the addition of the inhibiting ions on the active sites of the protein molecule rather than an exchange reaction with the active sites, causing the formation of some compounds of sulfur separate from the protein molecules. Considerable data exist on the addition reactions of bisulfite ions with dithio groups of cystine[8a] and with proteins containing dithio groups.[8b] The reaction investigated by Clarke was earlier suggested[7] as a possible mechanism of urease inhibition by bisulfite, but the quantitative data presented here make the suggestion improbable, already because the inhibition is too reproducible to be due to a primary oxidation of the enzyme by accidentally present oxidants. Urease is usually classed as a sulfhydryl enzyme, several agents which oxidize the sulfhydryl groups being known to inhibit this enzyme. The literature, however, fails to reveal information on reactions of the bisulfite ion with sulfhydryl groups and while their oxidation by this reagent cannot be entirely ruled out, it is at least not probable.

(7) I. W. Sizer and A. A. Tytell, *J. Biol. Chem.*, **138**, 631 (1941).

(8) (a) H. Clarke, *ibid.*, **97**, 235 (1932); (b) F. F. Ellsworth and H. Phillips, *Biochem. J.*, **35**, 135 (1941).

All of these considerations make an addition reaction more probable. Although the addition of the bisulfite ion to the carbonyl groups is well known, we cannot conclude that this is the case in the present investigation, as no other proof is available for the existence of the carbonyl group as a part of the active site of the enzyme molecule.

Summary

The bisulfite and the phenyl sulfinate ions inhibit reversibly the catalytic activity of urease, the inhibition being of first order in the concentration of the inhibitor. The inhibition reaction takes place only on those active sites which are not combined with urea. The heat of the reaction with the bisulfite is 9,200 cal./mole of the active sites and the standard entropy change is −21 e.u., which suggests that in this reaction bisulfite ion is added to the protein molecule. Sodium benzene sulfonate and sodium sulfate exert very much weaker inhibitory action. The oxidation–reduction potential of the sulfite–sulfate couple has no effect on the catalytic activity of crystalline urease.

CAMBRIDGE 38, MASSACHUSETTS
RECEIVED SEPTEMBER 19, 1949

[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Photochemical Decomposition of Ketene. II

BY G. B. KISTIAKOWSKY* AND N. W. ROSENBERG†

The photochemical decomposition of ketene has been studied by several investigators.[1] Their work has definitely established that the decomposition of ketene into carbon monoxide and methylene occurs as a primary reaction. The secondary reactions of the methylene are less clearly understood. The final products of irradiation in pure ketene are carbon monoxide and ethylene in approximately 2:1 ratio; the quantum yield of this reaction is on the order of unity. It has been suggested that the formation of ethylene occurs by the attack of methylene on ketene; an alternative suggestion is that the ethylene is produced by the association of two methylene diradicals. In the presence of ethylene another reaction occurs, leading to the formation of a non-volatile polymer. Careful analytical studies by Rosenblum[1f] have shown that these ideas on the mechanism of the over-all reaction can only be approximately correct in view of the presence of appreciable quantities of higher volatile hydrocarbons.

The original purpose of the present investigation was the measurement of the rate of association of methylene diradicals. The data obtained in preliminary work indicated that the association did not occur under the conditions employed; therefore a study was made of the reactions of methylene under these conditions, *i. e.*, irradiation of ketene and ketene–ethylene mixtures at pressures of the order of 100 mm. The nature of these reactions has been largely clarified, although several aspects of the mechanism remain unsettled.

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(1) (a) Norrish, Crone and Saltmarsh, *J. Chem. Soc.*, 1533 (1933); (b) Ross and Kistiakowsky, THIS JOURNAL, **56**, 1112 (1934); (c) Norrish, Crone and Saltmarsh, *ibid.*, **56**, 1644 (1934); (d) Pearson, Purcell and Saigh, *J. Chem. Soc.*, 409 (1938); (e) Burton, Davis, Gordon and Taylor, THIS JOURNAL, **63**, 1956 (1941); (f) Rosenblum, *ibid.*, **63**, 3322 (1941); (g) Norrish and Porter, *Disc. Far. Soc.*, **2**, 97 (1947).

Experimental Details

The reaction was studied in a static system utilizing radiation in the region 3000–3700 Å., largely that near 3100 Å. High radiation intensity and short illumination times avoided complications from dark reactions, notably the thermal polymerization of ketene. The progress of the reaction was studied manometrically and analytically; most runs were carried to less than five per cent. fractional decomposition.

The light source was of the type described by Taylor and Bates.[2] A low pressure mercury arc, it consisted essentially of two concentric quartz tubes. Through the central space was inserted the reaction cell, a 13-cm. length of 22-mm. o. d. Pyrex tubing with a wall thickness of 1.2 mm. and a volume of 40 cc. Water from a thermostat was forced through the annular space between the reaction cell and the inner lamp wall, maintaining the temperature of the reaction mixture constant to ±0.05°. This annular space also contained a 15-cm. length of aluminum tubing which could be moved back and forth, screening the reaction cell from radiation when desired. Capillary tubing led from the reaction cell to a wide-bore mercury manometer and to a capillary U-tube trap, from which, through a manifold, connections to reagent bulbs and to pumps were provided. The progress of the reaction was observed by measuring the change in the mercury level of the manometer by a microscope comparator; the reproducibility of the measurements was about 0.01 mm. A separate determination of the pressure of the condensible gases in the mixture was obtained by cooling the U-tube trap in liquid nitrogen and slowly pumping off the non-condensable fraction through it. After warming the residual gases, the pressure was measured by the microscope comparator. A Toepler pump permitted the withdrawal of samples for mass spectrographic analyses.

Ketene was prepared by the pyrolysis of acetone.[3] After passing through suitably cooled traps, it was condensed in the stillpot of a Podbielniak column 50 cm. long. Two fractionations of the sample with a stillhead temperature maintained constant at −78° by dry ice cooling resulted in a product containing no non-condensables (in liquid nitrogen) and less than 0.5% of impurities not absorbed in aqueous sodium hydroxide solution. Vapor density measurements indicated a molecular weight of 41.9 ± 0.1.

Ethylene, propylene and carbon dioxide were taken from high pressure cylinders and freed from non-condensable impurities by bulb-to-bulb distillation. A sample of

(2) Bates and Taylor, THIS JOURNAL, **49**, 2438 (1927).

(3) Hurd, *ibid.*, **45**, 3095 (1923).