repulsion between the molecules. The difference in second virial coefficients might also reflect a difference in binding of small ions by the two proteins.[27]

If β-lactoglobulin dissociates into the two equal subunits described by the Green and Aschaffenburg model,[28] the values of changes in entropies of rotation and translation can be estimated by the ideal gas equations.[28] The value obtained for their sum is *ca.* + 110 e.u. The difference between this value and the experimentally obtained value of 23–24 e.u. might be due possibly to a molecular rearrangement on dissociation which would introduce a higher degree of organization into the molecule or to a gain in water of hydration following dissociation. The immobilization of eight to ten water molecules per β-lactoglobulin submit would be sufficient to account for this. Since there is some evidence of appearance of a hydrophobic region on dissociation,[4] such an immobilization of water might occur by formation of clathrate[29] or ice-like[30] water structures. An alternate hypothesis is that the subunits of the protein are jointed by salt bridges which, upon rupture at low *p*H, allow water of electrostriction to be gained. A further contribution to the entropy term could arise from changes in proton binding during the dissociation.

The present experiments, although suggesting small differences between the dissociation patterns of the two β-lactoglobulins, leave unanswered questions on the structure of the subunits and on differences which may exist between them. Work has been initiated on this problem.

(27) S. N. Timasheff and B. D. Coleman, *Arch. Biochem. Biophys.*, **87** 63 (1960).

(28) See Glasstone, "Textbook of Physical Chemistry," 2nd Ed., D. Van Nostrand Company, New York, N. Y., page 873 ff.

(29) W. F. Claussen, *J. Chem. Phys.*, **19**, 1425 (1951); W. H. Rodebush and W. L. Masterson, *Proc. Natl. Acad. Sci., U. S.*, **40**, 17 (1954).

(30) I. M. Klotz, *Science*, **128**, 815 (1958).

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[CONTRIBUTION FROM THE PALO ALTO MEDICAL RESEARCH FOUNDATION, PALO ALTO, CALIFORNIA]

# The Urea Denaturation of Chymotrypsinogen as Determined by Ultraviolet Spectral Changes. Evaluation of Additional Kinetic Constants[1]

BY CHARLES H. CHERVENKA

RECEIVED MAY 17, 1960

The investigation of the *p*H dependence of rates of urea denaturation of chymotrypsinogen has been extended in order to study the nature of three unusually acidic groups previously found. By employing lower temperatures and urea concentrations, data are obtained which allow the calculation of all of the kinetic constants of the Levy and Benaglia formula. The apparent dissociation constants of the three groups are found to be near 2, and these are postulated to be "masked" carboxylic acids. Some thermodynamic quantities are estimated.

In a previous report of studies of the urea denaturation of chymotrypsinogen,[2] the results indicated the presence in the protein of three prototropic groups with unusually low *pK* values. These dissociation constants could not be evaluated at 25° because the denaturation reactions were too rapid to measure in media more acid than about *p*H 3. We now wish to report the results of another series of experiments, in which the rates were measured over the *p*H range from 1 to 9.

## Experimental

The methods and materials employed were the same as reported for the previous investigation.[2] Briefly, urea solutions containing hydrochloric acid or sodium hydroxide were equilibrated to the experimental temperature (± 0.1°) in the thermostated cuvette compartment of a Beckman model DU Spectrophotometer. A solution of five times recrystallized chymotrypsinogen was added at zero time, and the measured change in absorbance at 293 mμ with time was used to calculate first-order rate constants. All *p*H measurements were made at room temperature.

## Results

**Rates of Denaturation at Various Temperatures.**—Figure 1 illustrates the *p*H dependence of the apparent first order rate constants of the denaturation of chymotrypsinogen at various temperatures. From the trend of these results it was obvious that further decreases of temperature alone would not reduce the rates sufficiently to allow extension of the *p*H range lower than *p*H 3. However, by using lower urea concentrations at 9.5° the range was extended to *p*H 1, as shown in Fig. 2A, data from which are plotted as the logarithms of rate constants *versus* the logarithm of the activity of urea in Fig. 2B, using the activity coefficients for urea at 25°.[3] From the relationship[4]

$$n = \frac{\mathrm{d} \log \bar{k}}{\mathrm{d} \log U} \qquad (1)$$

the slopes of the lines in Fig. 2B give the apparent orders of reaction ($n$) with respect to the urea activity ($U$) at various *p*H values.

**Composite Rate Data.**—With these experimental values of the apparent order, eq. 1 was used to convert the rates of denaturation at various concentrations of urea to hypothetical values for 7.9 *M* urea. Fig. 3 shows the result of this computation and thus represents the variation in logarithm of the rate constant with *p*H from *p*H 1 to 9.

The special case of Levy and Benaglia's theory describing the *p*H dependence of denaturation rates[5] which was used previously[2] still applies to the new data at lower *p*H values; however, the

(1) This investigation was supported by research grant number A-2800 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service.

(2) C. H. Chervenka, THIS JOURNAL, **82**, 582 (1960).

(3) G. Scatchard, W. J. Hamer and S. E. Wood, *ibid.*, **60**, 3061 (1938).

(4) R. B. Simpson and W. Kauzmann, *ibid.*, **75**, 5139 (1953).

(5) M. Levy and A. E. Benaglia, *J. Biol. Chem.*, **186**, 829 (1950).

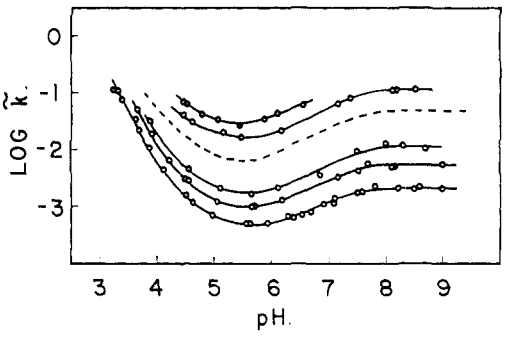


Fig. 1.—The $p$H dependence of the logarithm of first order rate constants for the urea denaturation of $1.6 \times 10^{-5}$ $M$ chymotrypsinogen at various temperatures. From top to bottom the curves are for 32.0, 28.8, 25.0, 18.9, 14.9, 9.5°; data for 25° (dashed line) are from ref. 2.

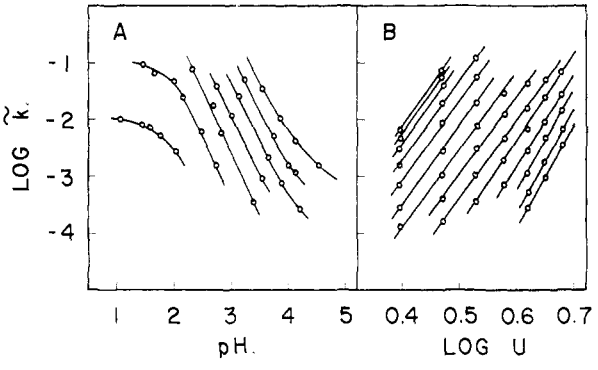


Fig. 2.—(A) The $p$H dependence of the logarithm of the first-order rate constants for the denaturation of $1.6 \times 10^{-5}$ $M$ chymotrypsinogen at 9.5° in various concentrations of urea. From right to left these concentrations are 7.9, 7.1, 6.3, 5.5, 4.8, 4.0 and 3.2 $M$. (B) Plot of the logarithms of the rate constants from $A$ *versus* the activity of urea at various values of $p$H. From right to left these $p$H values are 4.2, 4.0, 3.8, 3.6, 3.4, 3.2, 3.0, 2.8, 2.6, 2.4, 2.2, 2.0, 1.8 and 1.6.

full equation can be used now, and all the kinetic constants can be evaluated. This equation is

$$\bar{k} = \frac{\frac{k_1H^3}{K_1K_2K_3} + \frac{k_2H^2}{K_2K_3} + \frac{k_3H}{K_3} + K_4 + \frac{k_5K_4}{H}}{\frac{H^3}{K_1K_2K_3} + \frac{H^2}{K_2K_3} + \frac{H}{K_3} + 1 + \frac{K_4}{H}} \quad (2)$$

where $H$ represents hydrogen ion concentration, the $K$'s are the dissociation constants for the sequential ionization of four protons from the protein, and the $k$'s are the rate constants of denaturation of the resulting five ionic species. In the later discussions, the groups in the protein which have the dissociation constants $K_1$, $K_2$, etc., will be designated as $G_1$, $G_2$, etc. The values of the constants required to fit eq. 2 to the experimental data of Fig. 3, determined by the procedure described by Levy and Benaglia,[5] are given in Table I.

Thus of the four groups, the state of ionization of which influences the rate of denaturation, $G_1$ and $G_2$ have apparent $pK$ values of 1.95, $G_3$ has $pK$ 2.05 and $G_4$ has $pK$ 7.0, all in urea solution. Although the maximum slope of the curve in Fig. 1 does not reach −3 as might be expected from

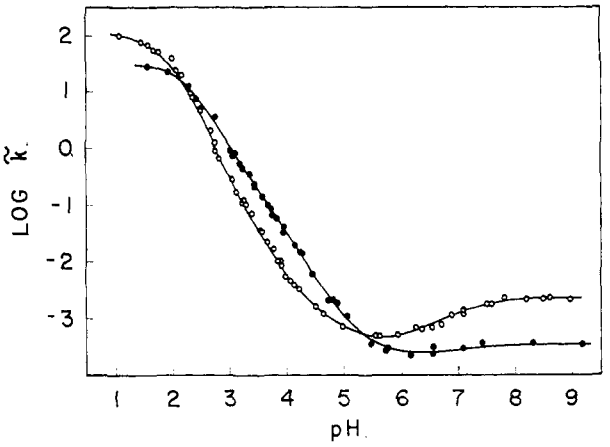


Fig. 3.—Composite plot of the variation of the rate constant with $p$H for $1.6 \times 10^{-5}$ $M$ chymotrypsinogen in urea at 9.5°: ○, no salt; ● 0.28 $M$ potassium chloride. The solid lines were calculated from eq. 2 in the text.

theory[6] and as was found for the data at 25°,[2] the curve cannot be fitted by assuming only two dissociation constants in the acid range. This failure of the slope to attain the theoretical value is due to overlapping of the rate regions for the different ionic species of the protein; for the same reason that the slope does not reach 1 between $p$H 6 and 8.

TABLE I

APPARENT KINETIC CONSTANTS FOR UREA DENATURATION AT 9.5°

| Constant | No added salt | Potassium chloride, 0.28 $M$ |
|---|---|---|
| $k_1$ (sec.$^{-1}$) | 112 | 33 |
| $k_2$ (sec.$^{-1}$) | 27.1 | 2.4 |
| $k_3$ (sec.$^{-1}$) | 0.34 | $3.8 \times 10^{-3}$ |
| $k_4$ (sec.$^{-1}$) | $3.03 \times 10^{-4}$ | $2.0 \times 10^{-4}$ |
| $k_5$ (sec.$^{-1}$) | $2.15 \times 10^{-3}$ | $3.7 \times 10^{-4}$ |
| $K_1$ (mole/l.) | $1.12 \times 10^{-2}$ | $6.3 \times 10^{-3}$ |
| $K_2$ (mole/l.) | $1.12 \times 10^{-2}$ | $6.3 \times 10^{-3}$ |
| $K_3$ (mole/l.) | $8.9 \times 10^{-3}$ | $8.5 \times 10^{-5}$ |
| $K_4$ (mole/l.) | $1.0 \times 10^{-7}$ | $1.0 \times 10^{-7}$ |

**Influence of Potassium Chloride.**—When the measurements of rates were repeated in the presence of 0.28 $M$ potassium chloride and the data treated as before, the composite curve shown in Fig. 3 was obtained. The empirical constants required to fit the data to eq. 2 are given in Table I. The rate constants are all decreased over those obtained in the absence of added salt. The values of $pK_1$ and $pK_2$ are increased moderately, as might be expected from the usual effect of ionic strength changes on dissociation constants of groups in a cationic region; however, $pK_3$ is increased by two units to 4.1, so that $G_3$ seems to have an unusual sensitivity to salt. In higher concentrations of potassium chloride little additional effect is noted. In 0.6 $M$ potassium chloride the rate $k_5$, given by the horizontal portion of the curve above $p$H 8 in Fig. 3, decreases slightly and approaches the value of $k_4$. In the acid region there is no change except that as the salt concentration is increased above 0.3 $M$ in the lower urea concentrations, a point is

(6) K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford University Press, London, 1958, p. 336.

reached where the protein precipitates, so that the spectral measurements can no longer be made.

**Effect of Urea on the Measurement of *p*H.**—The *pK* values given in this report are experimental quantities based on *p*H measurements in a medium which is considerably different from water. Since the activity of water is little affected by urea, *p*H values are obtained which are very nearly true[7]; however, apparent *pK*'s determined in urea are different from those in water.[8,9] For example, Levy[9] reported that the *pK* of acetic acid increased 0.5 unit in 7 *M* urea. We have found that $pK_1$ of glycine increases 0.6 unit in 7.9 *M* urea, while $pK_2$ of histidine increases 0.5 unit in this medium. The extent of change is directly proportional to the urea concentration. If it is assumed that the dissociation constants of protein groups are influenced in a comparable manner, then a rough correction of the values reported in Table I can be applied. Thus, in water the *pK* values for $G_1$, $G_2$ and $G_3$ would be near 1.7, and that of $G_4$ would be near 6.5.

**Thermodynamics.**—Since the rate data allow the calculation of $k_4$ and $k_5$ at different temperatures, the energy of activation of denaturation of two ionic species of chymotrypsinogen should be obtainable from the Arrhenius equation. However, the log *k versus* $1/T$ plot for $k_4$ is curved, and the energy of activation for this species appears to be a function of temperature. For $k_5$ the plot is linear above 12°; in this case a value for the energy of activation of 40 kcal. is obtained. At 25° the free energy of activation is calculated to be 19 kcal., and the entropy of activation is 65 e.u. Thus these functions have values typical of those of denaturation processes.

For rates determined in the presence of 0.28 *M* potassium chloride, these energy values are essentially the same.

To aid in the identification of $G_1$, $G_2$, $G_3$ and $G_4$, it would be desirable to determine the heats of ionization of these groups from the *pK* values obtained at different temperatures. Values for $G_4$ are uncertain, however, since the experimental solutions at neutral *p*H are essentially unbuffered and accurate determination of *p*H is difficult in this range. Thus there is no change in *p*H with temperature from 25 to 10° above the experimental error, which is of the order of ± 0.05 unit. Therefore it is doubtful that the decrease in $pK_4$ from 7.1 at 25°[2] to 7.0 at 9.5° is significant.

In the acid region where the *p*H measurements are more accurate, there is again no measurable change in *p*H with temperature, so that the heats of ionization of $G_1$, $G_2$ and $G_3$ are low or zero. Low values of heat of ionization would be expected for carboxylic acid groups.[10]

## Discussion

The apparent *pK* values reported here suggest that $G_1$, $G_2$ and $G_3$ are carboxylic acids with altered ionization properties; normal *pK* values for the $\beta$ or $\gamma$ substituents in proteins are from 4.0 to 4.7.[10] If these groups are carboxylic acids, then it is necessary to postulate a "masking" or "binding"[11,12] in order to account for the large shift in *pK*. Any of the concepts such as hydrogen bonding,[13,14] "frozen" water of hydration,[15] or "high-energy" covalent bonding[16] possibly could account for the changed dissociation constants, although the magnitude of the shift is such that hydrogen bonding alone is an unlikely explanation.[14,17]

In preliminary experiments, the results of which will be included in a later communication, acid titration data of denatured chymotrypsinogen give evidence to support the assumption of masked carboxylic acid groups in the native protein. For urea denatured chymotrypsinogen, even though the results are complicated by the shift of *pK* of all of the acidic groups, there is an increase in the slope of the titration curve between *p*H 3 and 5, indicating an increased proton uptake in this region.[12] Denaturation at *p*H 5 at 75° for three minutes yields a fine-grained suspension of protein which exhibits no *p*H drift during titration. In this case the titration curve is shifted from that of the native protein very nearly as would be expected if three groups underwent a change in *pK* from 1.7 to 4.0 during the denaturation process.

Since chymotrypsinogen has two tyrosine hydroxyl groups which are not free to ionize normally,[18] the possibility of tyrosyl-carboxylate hydrogen bonding must be considered,[11,13] although in general the experimental data do not support this idea. As mentioned, the shift in apparent *pK* values of the acid groups is too great for carboxyls in the usual type of tyrosyl-carboxylate bond; also, the anomalous behavior of the phenolic hydroxyl groups is not merely a shift in *pK* but is such that denaturation of the molecule must occur before any ionization of the two groups can take place.[18,19] Chymotrypsinogen does not exhibit the spectral shift in acid solutions which is typical of changes in the environment of tyrosine side chains (maximum change at 287 and 280 m$\mu$[19,20,21]). The spectral change in chymotrypsinogen solutions below *p*H 3[22] is more like that of tryptophan chromophores.[19,23]

As previously reported,[2] $G_4$ could be either an imidazolium or $\alpha$-ammonium group in the protein.

(7) H. Neurath, J. A. Rupley and W. J. Dryer, *Arch. Biochem. Biophys.*, **65**, 243 (1956).

(8) N. F. Burk and D. M. Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).

(9) M. Levy, *Compt. rend. trav. lab. Carlsberg. Sér. chim.*, **30**, 291 (1958).

(10) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press, Inc., New York, N. Y., 1958, p. 477.

(11) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(12) J. Steinhardt and E. M. Zaiser, *Advances in Protein Chem.*, **10**, 151 (1955).

(13) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. lab. Carlsberg. Sér. chim.*, **30**, 21 (1956).

(14) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

(15) I. M. Klotz, *Science*, **128**, 815 (1958).

(16) E. L. Smith, *J. Biol. Chem.*, **233**, 1392 (1958).

(17) D. B. Wetlaufer, *Compt. rend. trav. lab. Carlsberg. Sér. chim.*, **30**, 135 (1956).

(18) P. E. Wilcox, *Federation Proc.*, **16**, 270 (1957).

(19) C. H. Chervenka, *Biochim. Biophys. Acta*, **31**, 85 (1959).

(20) M. Laskowski, Jr., J. M. Widom, M. L. McFadden and H. A. Scheraga, *ibid.*, **19**, 581 (1956).

(21) D. B. Wetlaufer, J. T. Edsall and B. R. Hollingsworth, *J. Biol. Chem.*, **233**, 1421 (1958).

(22) C. H. Chervenka, Abstracts of Papers, Pacific Slope Biochemical Conference, Los Angeles, 1958.

(23) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *Biochim. Biophys. Acta*, **29**, 455 (1958).

The value of 6.5, which is obtained for the $pK$ of this group when the correction for the effect of urea is applied, falls into the range of normal values for imidazole side chains in proteins, 6.4 to 7.0,[10] and is far enough out of the range of normal values for $\alpha$-amino groups, 7.4 to 7.9,[10] that tentative identification of $G_4$ as an imidazolium group is reasonable.

Since salts reduce the rates of denaturation without increasing the activation energy, the influence of electrostatic forces, as discussed by Kauzmann,[24] must be considered in any explanation of the $p$H dependence of the rates of denaturation. It is obvious that the increases in rate on both sides of $p$H 5.7 are not due to the electrostatic repulsion of charges on the molecule as a whole; if this were the case the rate would be minimal at the isoelectric $p$H, which is above 9.[18] Also, the bonds holding the molecule together are probably not electrostatic, or the rate would increase at $p$H 5.7 upon the addition of salt. However, the possibility that electrostatic forces contribute to the weakening of critical bonds in a small portion of the molecule at $p$H values on either side of 5.7 is not eliminated. Such a portion of the molecule would correspond to the "critical seam" in the theory of London, *et al.*[25]

It is interesting to note that Eisenberg and Schwert found that the extent of reversible heat denaturation of chymotrypsinogen is related to the ionization of three acid groups.[26] It would seem reasonable that these are the same groups found to be critical in the urea denaturation. Since the mechanism of the two types of denaturation are not necessarily the same and since the values for heat denaturation were determined under very different conditions and from equilibrium constants rather than rate constants, the difference in apparent $pK$ values found ($pK$ 2.5 found for heat denaturation) is not surprising.

ADDED IN PROOF.—After this work was accepted for publication a private communication was received from Dr. P. E. Wilcox describing the results of prior experiments on the titration of denatured chymotrypsinogen. These data show that after denaturation in 4 $M$ guanidinium chloride three additional protons are taken up by the protein in the $p$H region of carboxylic acid ionization.

(24) W. Kauzmann, in W. D. McElroy and B. Glass, "The Mechanism of Enzyme Action," The Johns Hopkins Press, Baltimore, Md., 1954, p. 70.

(25) M. London, R. McHugh and P. B. Hudson, *Arch. Biochem. Biophys.*, **73**, 72 (1958).

(26) M. A. Eisenberg and G. W. Schwert, *J. Gen. Physiol.*, **34**, 583 (1951).

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[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

# The Sedimentation Behavior of Human Pituitary Growth Hormone

BY PHIL G. SQUIRE AND KAI O. PEDERSEN[1]

RECEIVED JULY 15, 1960

An attempt to determine the molecular weight of human pituitary growth hormone was complicated by the fact that molecular association of the protein occurred between $p$H 2 and 10. Sedimentation velocity experiments, performed at protein concentrations ranging from 0.53 to 36.0 g./l. in a phosphate buffer of $p$H 2.38 revealed in addition to the major component the presence of material with a higher sedimentation coefficient. The amount of rapidly sedimenting material increased with total protein concentration, and the weight average sedimentation coefficient calculated from the rate of movement of the second moment of the entire schlieren diagram also had a strong positive slope, both observations indicating an association that is dependent upon concentration. Values for the molecular weight of the hormone were calculated from studies of the approach to equilibrium (Archibald procedure). The values calculated from the top and bottom menisci did not follow the same smooth curve when plotted as functions of concentration, suggesting that the reaction rate was slow compared with the time of the experiment (1–2 hr.). The best values for the molecular weight of the monomer were obtained by extrapolating the upper miniscus values to infinite time and by extrapolating the zero time values to infinite dilution. Both methods give a value of about 29,000 as the molecular weight of the hormone.

## Introduction

Marked biological and physicochemical differences have been demonstrated between growth hormone preparations obtained from bovine and human pituitaries.[2,3] Preliminary investigations of the molecular weight of human growth hormone have been reported from three different laboratories. The results of Ehrenberg and Heijkenskjöld[4] obtained by sedimentation velocity, and Ehrenberg's application[5] of the Archibald procedure, showed that the sample was quite heterogeneous. These investigators drew the conclusion that the molecular weight was 15,000–20,000, but they pointed out that the sample also contained material of higher molecular weight. Li and Papkoff[6] reported sedimentation velocity experiments carried out in a phosphate buffer of $p$H 2.3, in which a sedimentation coefficient of 2.47 S was obtained. Using this value and a diffusion coefficient determined from an independent experiment in the same buffer, they calculated a molecular weight of 27,100. The sedimentation velocity studies of Lewis and Brink[7] yielded results which were substantially in agreement with those reported by Li and Papkoff.[6]

The availability of additional material has permitted a more extensive study of the molecular weight than had been possible previously. These studies have demonstrated that the hormone undergoes a reversible association reaction, as evidenced by sedimentation behavior. The molecular weight of the hormone has been determined by the Archibald procedure.

(1) University of Uppsala, Sweden.

(2) C. H. Li, *Federation Proc.*, **16**, 775 (1957).

(3) C. H. Li, H. Papkoff and C. W. Jordan, Jr., *Proc. Soc. Exptl. Biol. Med.*, **100**, 44 (1959).

(4) A. Ehrenberg and F. Heijkenskjöld, *Acta Chim. Scand.*, **10**, 1675 (1956).

(5) A. Ehrenberg, *ibid.*, **11**, 1257 (1957).

(6) C. H. Li and H. Papkoff, *Science*, **124**, 1293 (1956).

(7) U. J. Lewis and N. G. Brink, THIS JOURNAL, **80**, 4429 (1958).