sis of acetyl-L-tyrosinamide by two representative bifunctional anionic competitive inhibitors in aqueous solutions at 25° and *p*H 7.9 ± 0.1 in the presence of varying amounts of potassium phosphate.

The authors wish to express their indebtedness to Drs. Robert Bock, Ralph Lutwack and Myron Arcand for many helpful suggestions and criticisms offered during the course of this investigation.

Experimental[23]

Specific Substrate and Competitive Inhibitors.—Acetyl-L-tyrosinamide, colorless needles, m.p. 226–228°, $[\alpha]^{24}D$ + 51.8° (*c* 0.8%, in water) was prepared as described previously.[9] β-(β-Indole)-propionic acid, colorless needles, m.p. 133–134°, was recrystallized twice from a mixture of water and methanol. β-(β-Indole)-propionamide, fine, stunted colorless needles, m.p. 205–207°, was prepared as described previously.[4] Phenylacetic acid, shiny platelets, m.p. 77–78°, was recrystallized three times from a mixture of ethanol and water. Phenylacetamide, short, colorless needles, m.p. 157–158°, was prepared as described previously.[4] Tryptamine hydrochloride, short dense colorless prisms, m.p. 250–251°, was recrystallized twice from aqueous 5 *N* hydrochloric acid.

Enzyme Experiments.—The analytical procedure described previously[9] was employed without modification. The anionic competitive inhibitors were introduced into the reaction mixtures in the form of their potassium salts. The buffer compounds were prepared as before.[9] Crystalline bovine α-chymotrypsin Armour lot no. 00592 was employed in all experiments. The primary data were evaluated as before.[9]

(23) All melting points reported are corrected.

PASADENA, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, WEIZMANN INSTITUTE OF SCIENCE, AND THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

The Availability of the Disulfide Bonds of Human and Bovine Serum Albumin and of Bovine γ-Globulin to Reduction by Thioglycolic Acid

BY EPHRAIM KATCHALSKI, GEORGE S. BENJAMIN AND VIOLET GROSS

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The extent of reduction of the disulfide bonds of human and bovine serum albumin and of bovine γ-globulin by thioglycolic acid at different *p*H values was investigated. Upon reduction human and bovine serum albumin acquired a maximum of one thiol group per molecule in the *p*H range 5.0 to 7.0. Beyond this range an increasing number of disulfide bonds became susceptible to reduction. In the case of bovine γ-globulin a maximum of one thiol group per molecule appeared upon reduction in the range of *p*H 3.5 to 5.0. With an increase in *p*H an increasing number of disulfide bonds could be reduced. The effect of *p*H on the number of disulfide bonds available for reduction was found to be reversible for bovine serum albumin and for bovine γ-globulin in the *p*H ranges of 1.2 to 10.2 and 5.0 to 10.2, respectively. In the three proteins investigated reduction of most of the disulfide bonds could be effected at *p*H values above 10 in the presence of guanidine. The conversion of unreactive disulfide bonds into reactive ones is explained as being due to changes in the configuration of the protein molecule, which may be either reversible or irreversible ones.

In contrast to the extensive literature dealing with the chemical reactivity of the thiol groups of native and denatured proteins,[1] only a few investigations on the reactivity of the disulfide groups of proteins have been published. Most of the work on the chemical reactivity of protein disulfide groups is concerned with their reduction which can be performed under mild experimental conditions. Reductions of the disulfide bonds of insulin,[2] keratin[3] and lactogenic hormone[4] have been reported. In the case of egg and serum albumin[5] and insulin[6] it was demonstrated that complete reduction of the disulfide bonds by thioglycolic acid can only occur after denaturation. The number of detectable –SH and S–S groups were found by Mirsky and Anson[7] to be closely linked with the extent of denaturation. On reversal of denaturation the number of detectable S–S groups decreased.

In the present article the reduction of the disulfide bonds of human and bovine serum albumin and of bovine γ-globulin by thioglycolic acid at different *p*H values will be described. The results obtained suggest that reversible as well as irreversible changes in the configuration of these proteins may occur.

Experimental

Materials.—Human serum albumin was prepared according to Cohn, *et al.*,[8] and crystallized three times in the presence of decanol. Its thiol content, determined by titration with methyl mercury nitrate (see below), was about 0.3 mole –SH per mole of albumin, assuming 65,000 as the molecular weight of the protein.[9] For the discussion below the value of 17 cystine residues per serum albumin molecule reported by Hughes[9] will be accepted.

Mercaptalbumin.—Mercury–mercaptalbumin dimer was prepared according to Hughes[10,11] and recrystallized five times before use. A pure aqueous solution of mercaptalbumin monomer was obtained from the mercury dimer according to the procedure of Dintzis.[12,13] Titration of the resulting concentrated mercaptalbumin solution with

(1) *Cf.* E. S. G. Barron, *Advances in Enzymology*, **11**, 201 (1951); H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944); F. W. Putnam, H. Neurath and K. Bailey, Editors, "The Proteins," Vol. IB, Academic Press, Inc., New York, N. Y., 1954, p. 807.

(2) V. du Vigneaud, A. Fitch, E. Pekarek and W. W. Lockwood, *J. Biol. Chem.*, **94**, 233 (1931); K. G. Stern and A. White, *ibid.*, **117**, 95 (1937).

(3) D. R. Goddard and L. Michaelis, *ibid.*, **112**, 361 (1935).

(4) H. Fraenkel-Conrat, M. E. Simpson and H. M. Evans, *ibid.*, **142**, 107 (1942); H. Fraenkel-Conrat, *ibid.*, **142**, 119 (1942).

(5) A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **18**, 307 (1935).

(6) H. Lindley, THIS JOURNAL, **77**, 4927 (1955).

(7) A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **19**, 427 (1936).

(8) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(9) *Cf.* W. L. Hughes: H. Neurath and K. Bailey, Editors, "The Proteins," Vol. IIB, Academic Press, Inc., New York, N. Y., 1954, p. 663.

(10) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947).

(11) W. L. Hughes, Jr., *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 79 (1949).

(12) H. M. Dintzis, Thesis, Harvard University, 1952.

(13) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

methyl mercury nitrate (see below) indicated that the mercaptalbumin regenerated contained 0.95 to 1.00 sulfhydryl groups per protein molecule assuming a molecular weight of 65,000.

Bovine serum albumin was obtained from Armour Laboratories, Lot No. 66706, and found to contain 0.25 to 0.35 sulfhydryl group per molecule assuming a molecular weight of 65,000.[9] Also in this case the presence of 17 cystine residues per molecule will be assumed.[9]

Bovine γ-globulin was obtained from Armour Laboratories, Lot B. P. 201-204. No sulfhydryl groups could be detected in this protein on titration with methyl mercury nitrate. The analytical data of Smith and Greene[14] show that the protein contains 18 cystine residues per molecule assuming a molecular weight of 150,000.[9]

Thioglycolic acid was obtained from Eastman Kodak Co. and distilled *in vacuo* before use.

Guanidine Reagent.—Concentrated hydrobromic acid (48%) was added to solid guanidine carbonate until the *p*H was neutral to Hydrion paper. The mixture was filtered and brought to *p*H 10 to 11 with anhydrous sodium carbonate. Solid ethylenediaminetetraacetic acid (Sequestrene, Alrose Chemical Co.) was added to make the solution 0.01 molar in this substance. The ethylenediaminetetraacetic acid was included to bind any traces of heavy metal ions which interfere with the nitroprusside reaction.[11] The final concentration of the guanidine hydrobromide was approximately 5 *M*.

Methyl Mercury Nitrate Solution.—Methyl mercury iodide was crystallized three times from ethanol and a weighed amount of the purified material dissolved in a minimal amount of 95% ethanol. The solution was mixed with an equivalent amount of silver nitrate which had been dissolved in a small volume of water. The precipitated silver iodide was filtered and washed thoroughly with water. The filtrate and washings were combined and diluted with water to give a 10^{-3} *M* methyl mercury nitrate solution. The 10^{-3} *M* methyl mercury nitrate solution could be stored without change for several months in a brown bottle at 0°.

Methods. Reduction of Protein Disulfide Bonds.—Protein solutions of approximately 8% were prepared. Water was used as solvent for human and bovine serum albumin and 2% aqueous sodium chloride for bovine γ-globulin. The desired *p*H was obtained by the addition of 0.1 *N* hydrochloric acid for values below the isoelectric point, of 4% aqueous sodium carbonate for values between the isoelectric point and *p*H 10, or of 1 *N* sodium hydroxide for *p*H values above 10. Protein concentration was then determined by Kjeldahl nitrogen analysis.

The protein solution (5 ml.) was mixed in a Thunberg tube with 0.2 *M* aqueous thioglycolate (5 ml.), previously adjusted to the *p*H of the protein solution, and with water (10 ml.). The resultant *p*H did not differ significantly from that of the component solutions. The reaction mixture was frozen in an ice-salt-bath and the tubes were evacuated and filled with oxygen-free nitrogen. This procedure was repeated three times to ensure complete removal of oxygen. The reduction was finally allowed to proceed at 0°. When the reduction was carried out in the presence of guanidine, a 5 *M* guanidine hydrobromide solution (10 ml.), previously adjusted to the required *p*H, was added to the protein–thioglycolate mixture instead of water.

The final reduction mixtures contained approximately 10 to 20 molecules of reducing agent per protein disulfide bond. The data of Bersin and Steudel[15] for the reduction of cystine seem to indicate that the excess of reducing agent used is adequate for the complete reduction of all available protein disulfide links in the *p*H range investigated.

Titration of Protein -SH Groups.—Sufficient protein to contain approximately 0.5 micromole -SH (*e.g.*, 50 mg. of albumin) was dissolved in 2 ml. of cold guanidine hydrobromide reagent. (Some proteins including serum albumin coagulate as a gel when treated in the dry state with concentrated guanidine salt solution. In such cases, it was advantageous to dissolve the protein first in a small amount, 0.5 ml. or less, of water or diluted guanidine reagent.) A drop of 10% $Na_4Fe(CN)_5NO$ was added and the solution was then titrated with 10^{-3} *M* methyl mercury nitrate to the disappearance of the purple color. The titration was carried out in the cold near 0°. If the solution was not properly cooled or if the titration was unnecessarily prolonged, the end-point was confused by a persistent orange or yellow color. The titration values obtained for a given sample did not deviate by more than 5% from their average.

The protein present in the reduction mixture was prepared for SH- analysis as follows: To an aliquot (2 ml.) of the reduction mixture, 3% trichloroacetic acid (10 ml.) was added and the precipitated protein was centrifuged. The supernatant containing excess thioglycolic acid was discarded and the precipitate resuspended in 3% trichloroacetic acid (10 ml.). The protein was again centrifuged and the procedure repeated five times. The final supernatant contained no thiol groups and the sulfhydryl content of the protein precipitated remained unaltered on further washing with 3% trichloroacetic acid.

Results

Reduction of Human Serum Albumin.—The course of reduction of human serum albumin at different *p*H values in the range of *p*H 3.0 to 10.0 is given in Fig. 1. The figure shows that at each

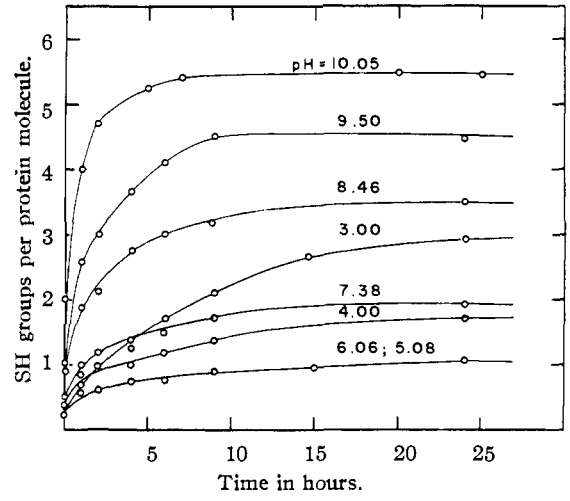

Fig. 1.—Course of reduction of human serum albumin at different *p*H values.

of the *p*H values investigated the number of thiol groups acquired per albumin molecule approached a final value within 24 hr. This value as a function of *p*H is given in Fig. 2, curve 1. The protein reduced in the *p*H range 5.0 to 7.0 was found to contain only one thiol group per molecule. Decreasing or increasing the *p*H of reduction beyond this range resulted in an increase in the number of thiol groups per molecule, the increase being greater in the alkaline range.

When mercaptalbumin was treated with thioglycolic acid in the *p*H range 5.0 to 7.0, no thiol groups in addition to the one originally present per molecule appeared. Furthermore a plot of the data obtained for mercaptalbumin over the whole *p*H range 3.0 to 10.0 gave a curve identical with that obtained with human serum albumin (Fig. 2, curve 1).

Reduction of human serum albumin in the presence of guanidine at *p*H 10.3 caused the appearance of 30 to 31 thiol groups per molecule as compared to the appearance of only 6 thiol groups per molecule at *p*H 10.0 in the absence of guanidine.

In order to ascertain whether the excess of thioglycolate used was sufficient to reduce all the susceptible disulfide bonds, reductions were carried out at *p*H 4.2, 6.0 and 8.45, where the thioglycolate

(14) E. L. Smith and R. D. Greene, *J. Biol. Chem.*, **171**, 355 (1947).
(15) Th. Bersin and I. Steudel, *Ber.*, **71**, 1015 (1938).

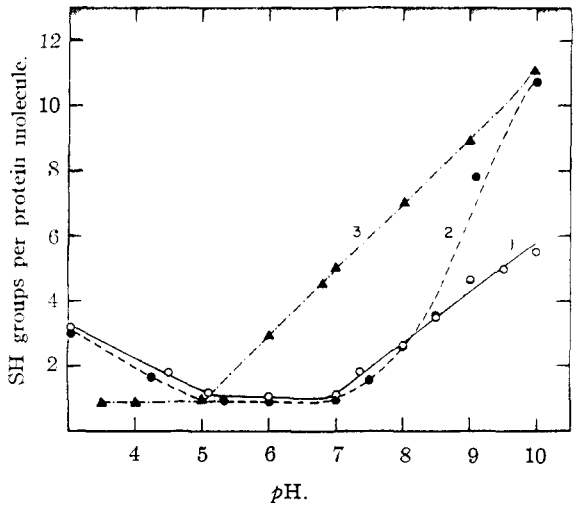

Fig. 2.—Number of thiol groups acquired in 24 hr. as a function of pH by: human serum albumin (—○—○—), bovine serum albumin (- - -●- - -●- - -) and bovine γ-globulin (·—▲·—·—▲·—).

concentration was maintained at 0.05 M and the protein concentration varied between 0.46 and 3.7%. The number of thiol groups acquired within 24 hr. per protein molecule at any of the pH values investigated was found to be practically independent of the relative excess of the reducing agent.

Reduction of Bovine Serum Albumin.—The course of reduction of bovine serum albumin in the pH range 3.0 to 8.5 proceeded similarly to that of human serum albumin. The final number of thiol groups acquired by both proteins in this pH range was also similar. In the pH range 8.5 to 10.0, however, bovine serum albumin acquired a considerably larger number of thiol groups than human serum albumin (see Fig. 2, curve 2). In the presence of guanidine approximately 26 thiol groups were acquired by bovine serum albumin upon reduction at pH 11.0. Denaturation of the protein leads therefore, as in the case of human serum albumin, to the exposure of most of the protein disulfide bonds to reduction.

To study whether the effect of pH on the availability of disulfide bonds to reduction is reversible, the following experiments were performed. Solutions of bovine serum albumin of various hydrogen ion concentrations were allowed to stand at 0° for different periods of time. The pH was then adjusted to 6.0 and the protein reduced with thioglycolate as already described. It was found (Table I) that samples of bovine serum albumin kept at pH ranges 1.2 to 3.5 and 8.1 to 10.2 acquired on reduction approximately one thiol group per albumin molecule. As the same number of thiol groups was obtained upon the reduction of freshly prepared protein solutions at pH 6.0, it can be concluded that the changes in the albumin molecule in the above acidic and basic ranges are reversible. Samples which were kept at pH 13.0 for as little as 1.5 hr. acquired on reduction at pH 6.0 four SH-groups per albumin molecule. This indicates that at pH 13.0 an irreversible change occurred. A similar irreversible change, but to a somewhat lesser extent, occurred at pH 11.0 and 11.9.

TABLE I

THE EXTENT OF REDUCTION OF BOVINE SERUM ALBUMIN AT pH 6.0 AFTER PRIOR EXPOSURE TO DIFFERENT pH VALUES

pH Prior to reduction[a]	Time of exposure, hours	SH/protein molecule after reduction[b]
1.2	24	0.85
1.7	24	.90
2.35	24	.94
3.5	24	.98
8.1	24	.95
9.0	24	.99
9.6	2.5	.95
10.2	24	1.00
11.0	2	0.97
11.0	6.5	1.00
11.0	24	1.35
11.9	3	1.10
11.9	24	1.80
13.0	1.5	4.03
13.0	6.0	4.80

[a] Concentration of albumin 6.5 to 7.0% (w./v.). [b] The reduction mixture contained approximately 2% albumin and thioglycolic acid at a 0.05 M concentration. The values given were obtained after reduction at pH 6.0 for 24 hr.

Reduction of Bovine γ-Globulin.—The rate of reduction of bovine γ-globulin was as a rule somewhat faster than that of human or bovine serum albumin. The maximum number of thiol groups acquired on reduction with thioglycolic acid at different pH values is recorded in Fig. 2, curve 3. In the pH range 5.0 to 10.0 the number of protein -SH groups increased almost linearly with pH. In the pH range 3.5 to 5.0 only one thiol group per protein molecule was found. At pH 10.0 in the presence of guanidine hydrobromide 20 to 22 sulfhydryl groups per γ-globulin molecule appeared.

Experiments to study the reversibility of the exposure of disulfide bonds of bovine γ-globulin to reduction analogous to those carried out with bovine serum albumin were performed. The protein was kept in solution at 0° for 24 hr. at pH values in the range of pH 5.0 to 12.0. The pH of the aqueous solutions was then adjusted to pH 5.0 and the reduction with thioglycolate carried out as usual. The samples thus treated in the pH range 5.5 to 10.2 acquired on reduction at pH 5.0 approximately one thiol group per globulin molecule. The exposure of disulfide bonds of γ-globulin in the above pH range is therefore reversible. Bovine γ-globulin samples exposed to pH 12.0 for only 2 hr. acquired on reduction at pH 5.0, 3.5 SH-groups per molecule. indicating that an irreversible change occurred.

Discussion

The results reported above show that each of the three proteins investigated is characterized by a pH range at which no significant reduction of disulfide bonds by thioglycolic acid takes place. Beyond this region the number of disulfide bonds susceptible to reduction increases. The change in reactivity of the protein disulfide groups toward thioglycolic acid may be explained by assuming a change in the shape and configuration of the protein mole-

cule transforming chemically unreactive groups to chemically active ones.

Extensive unfolding of the peptide chains of native proteins caused by the breaking up of intramolecular hydrogen bonds is known to take place in the presence of denaturing agents such as urea and guanidine.[1] Such a far reaching change in the configuration of the protein molecule would explain the result obtained where in the presence of guanidine at *p*H 10 most of the disulfide bonds of each of the three proteins investigated were reduced.

The respective *p*H ranges at which human and bovine serum albumin and bovine γ-globulin were found to have a minimal number of disulfide bonds available for reduction correspond to those *p*H ranges where these proteins are known to have a relatively small net electrical charge. Increasing the acidity or alkalinity beyond this *p*H range led to an increase in the number of disulfide bonds reduced. This suggests that increasing the net electrical charge leads to some unfolding of the protein molecule due to electrostatic repulsion thereby exposing a few disulfide bonds to reduction. The observed reversal of the number of disulfide bonds available for reduction implies that the change in configuration of bovine serum albumin in the *p*H range 1.2 to 10.2 (see Table I) and of bovine γ-globulin in the *p*H range 5.0 to 10.2 are reversible.

Similarly, other investigators have concluded that bovine serum albumin can undergo reversible configurational changes within limited *p*H ranges. Klotz, *et al.*,[16] observed that the binding of anionic dyes by this protein is reversible and increases from *p*H 6.8 to 9.2. This was explained as being due to a reversible change in the intramolecular bonding of the protein leading to the exposure of new side chains capable of interaction within the specific dye molecules. Tanford[17] found that the electrostatic interaction factor w decreases for bovine serum albumin from *p*H 5.0 to 2.5. The decrease in w was interpreted to be the result of a corresponding increase in the radius of the molecule. Champagne and Sadron[18] measured the viscosity and diffusion of bovine serum albumin over the *p*H range 3.5 to 10.0. From the data obtained they concluded that the shape of the molecule undergoes a reversible change from a sphere at its isoelectric point, *p*H 5.3, to an elongated ellipsoid of revolution in the *p*H ranges 5.3 to 3.45 and 5.3 to 7.4.

The curves in Fig. 2 show that bovine γ-globulin in contrast to human and bovine serum albumin already exists in a partially unfolded state at physiological *p*H. This may somehow be related to the immunological functions of the γ-globulins.

(16) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, *J. Phys. Chem.*, **56**, 77 (1952).

(17) C. Tanford: T. Shedlovsky, Editor, "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955, p. 248.

(18) M. Champagne and C. Sadron, "Simposio Internazionale di Chimica Macromoleculare," Supplemento a "La Ricerca Scientifica," Anno 25°, 1955, p. 3.

REHOVOTH, ISRAEL

[CONTRIBUTION FROM THE WEIZMANN INSTITUTE OF SCIENCE]

The Carbonyl Absorption of Carbamates and 2-Oxazolidones in the Infrared Region

BY S. PINCHAS AND D. BEN-ISHAI

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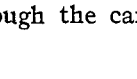
The data for the C=O frequency in 21 carbamates show that each type of a carbamic group has its own characteristic absorption region. Primary carbamates, both in the solid phase and in solution in chloroform, absorb at 1725 ± 3 cm.$^{-1}$. Secondary carbamates absorb at 1705–1722 and tertiary at 1687 ± 4 cm.$^{-1}$. The C=O frequency of the carbamic group in linear carbamates with a cyclic nitrogen atom depends upon the electronic effects of the ring and its substituents. Fourteen 2-oxazolidones showed their cyclic carbamic C=O frequency at 1746–1810 cm.$^{-1}$. An appreciable absorption band at 1029–1059 cm.$^{-1}$ is characteristic for the 2-oxazolidone ring.

Although the carbonyl absorption band of the



carbamates, $R_1R_2N\text{-}\overset{\overset{O}{\|}}{C}\text{-}OR_3$, has already been reported to appear in the 1690–1736 cm.$^{-1}$ region,[1] only two exact values for this frequency in the case of simple carbamates could be found in the literature[2] these being 1661 cm.$^{-1}$ for an oil paste of ethyl carbamate (urethan) and 1706 cm.$^{-1}$ for a liquid layer of ethyl N-methylcarbamate (methylurethan). Recently the carbonyl frequency of various esters of phenylcarbamic acid (phenylurethans) was measured[3] and was also found to be in this region being about 40 cm.$^{-1}$ higher than in anilides.

Since simple amides can usually be recognized as primary, secondary or tertiary amides from the frequency of their C=O stretching vibration in dilute non-polar solutions, this frequency appearing at about 1690, 1680 and 1650 cm.$^{-1}$, respectively,[4] it was interesting to see whether a parallel regularity could also be found for the similar carbamates. In view of the already mentioned lack of data for the carbonyl frequency in individual simple carbamates, their measurement seemed worth while and, since a series of such compounds was at our disposal, this measurement was undertaken.

Table I summarizes the results obtained for the carbamic carbonyl group frequency in 21 various carbamates.

(1) (a) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," Methuen & Co., London, 1954, p. 191; (b) H. Thompson, D. Nicholson and L. Short, *Discussions Faraday Soc.*, **9**, 229 (1950).

(2) H. M. Randall, R. G. Fowler, N. Fuson and J. R. Dangl, "Infrared Determination of Organic Structures," D. Van Nostrand Co., Inc., New York, N. Y., 1949, pp. 157–159.

(3) (a) N. F. Hayes, R. H. Thomson and M. St. C. Flett, *Experientia*, **11**, 61 (1955); (b) D. A. Barr and R. N. Haszeldine, *J. Chem. Soc.*, 3428(1956).

(4) R. E. Richards and H. W. Thompson, *J. Chem. Soc.*, 1248 (1947).