[CONTRIBUTION FROM THE DEPARTMENTS OF PHYSIOLOGICAL CHEMISTRY AND CHEMISTRY, UNIVERSITY OF WISCONSIN]

Effect of Cysteine–Hydrogen Peroxide Treatments on the Electrophoretic and Immunological Assay Behavior of Human Gamma Globulin[1]

BY H. F. DEUTSCH

The digestion of human gamma globulin by pepsin gives hydrolytic cleavage products which have essentially the same electrophoretic mobility as the undigested material.[2] On the other hand, it appears that the digestion of gamma globulin with papain and bromelin gives molecular fragments the greater portion of which have higher electrophoretic mobilities at a given *p*H than does the parent molecule.[3] Using undigested and pepsin digested human gamma globulin it was found that in the presence of cysteine and hydrogen peroxide, the substances used to activate and inactivate the above plant proteases, these proteins acquired higher electrophoretic mobilities and became more heterogeneous. Moreover, after such chemical treatment the antibody titers of these proteins decreased markedly.

Experimental

Methods.—The gamma globulin used in the experiments was the "so-called" Fraction II of Cohn, *et al.*[4] It was prepared by alcohol fractionation of human plasma[4a] and was 97% pure on electrophoretic analysis. The pepsin digested globulin was the Fraction DS2–4 prepared in our earlier work.[2] To 5% solutions of these proteins in 0.15 molar sodium chloride was added 100 mg. of cysteine hydrochloride per g. of protein. The *p*H of the solutions was adjusted to *p*H 7.0 (±0.1) with 0.05 molar disodium phosphate. Such solutions were dialyzed against 0.3% hydrogen peroxide for varying periods of time. Samples for electrophoretic analysis were diluted 1–1 with a *p*H 8.6 buffer that was 0.05 *M* in sodium diethylbarbiturate and 0.00765 *M* in sodium citrate (ionic strength = 0.088). Such samples were then dialyzed for thirty-six to forty-eight hours against several changes of this buffer. The duration of the electrophoretic experiments was two hours, with a constant potential gradient of approximately 10 volts per cm. being used. The diagonal knife-edge schlieren method was used to record the moving boundaries. Mobilities were measured on the descending side, using the salt boundary as the reference point.

Ultracentrifuge analyses were performed in the oil-turbine high velocity ultracentrifuge of Svedberg at 225,000 times gravity, using the schlieren method to record each molecular mass spectrum.

The material for immunological assay containing 100 mg. of cysteine hydrochloride per g. of protein was dialyzed against 0.3% hydrogen peroxide for eighteen hours. Following dialysis against repeated changes of 0.15 molar sodium chloride solution the samples were frozen and dried *in vacuo*. The dry proteins were then reconstituted into solution as desired for immunological assay.

Results

No changes were noticed in the electrophoretic mobility of the pepsin digested gamma globulin following dialysis against 0.3% hydrogen peroxide or upon direct addition of hydrogen peroxide to give a concentration of 1%. When this protein was allowed to stand in the presence of cysteine (100 mg. per g. of protein) for forty-eight hours a definite increase in heterogeneity was noticed. However, the addition of hydrogen peroxide to a level of 1% to a digested globulin system containing 50 mg. each of cysteine hydrochloride and sodium cyanide per g. of protein caused an increase in the electrophoretic mobility from 1.5×10^{-5} to 5.6×10^{-5} sq. cm./volt/sec. The electrophoretic pattern of digested gamma globulin so treated as compared with the control and cysteine treated material is shown in Fig. 1.

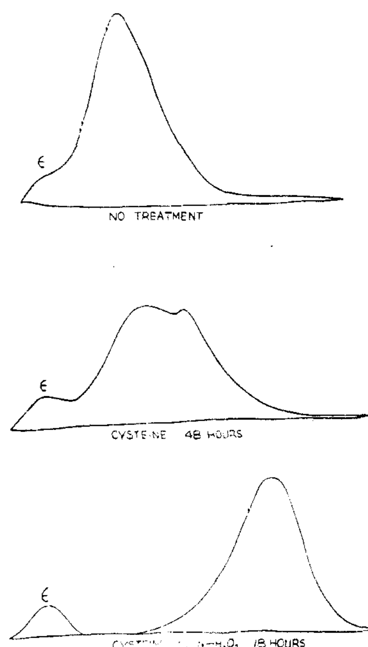

Fig. 1.—Effect of cysteine and of cysteine–sodium cyanide–hydrogen peroxide treatment on the electrophoretic patterns of pepsin digested gamma globulin. Duration of electrophoresis experiment for A and B was 120 minutes, C was 89 minutes with a potential gradient of approximately 10 volts/cm.

Dialysis of 5% solutions of gamma globulin which had contained cysteine for varying periods of time against 0.3% hydrogen peroxide in 0.15 molar sodium chloride gave protein systems which showed constant increases in electrophoretic mobility. In Figs. 2A and 2B it can be seen

(1) The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Wisconsin.

(2) H. F. Deutsch, M. L. Petermann and J. W. Williams, *J. Biol. Chem.*, **164**, 93 (1946).

(3) M. L. Petermann, THIS JOURNAL, **68**, 106 (1946).

(4) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H. Armstrong, *J. Clin. Invest.*, **23**, 417 (1944); E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(4a) H. F. Deutsch, L. J. Gosting, R. A. Alberty and J. W. Williams, *J. Biol. Chem.*, **64**, 109 (1946).

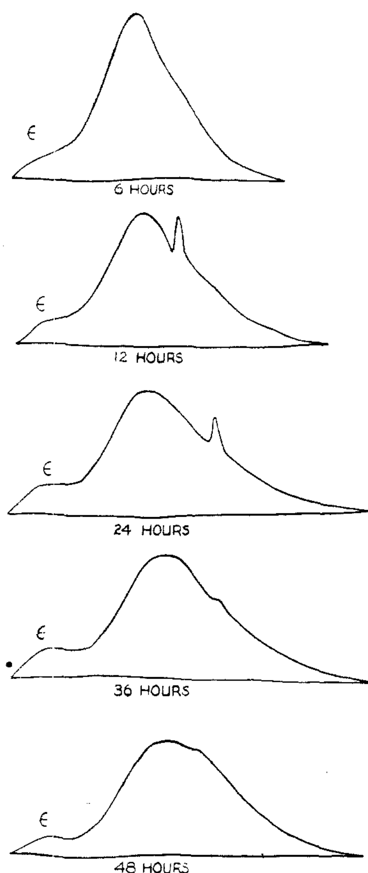

Fig. 2A.—Effect of dialysis of pepsin digested gamma globulin containing cysteine against 0.3% hydrogen peroxide for varying periods of time. Duration of electrophoresis experiments was 120 minutes at approximately 10 volts/cm.

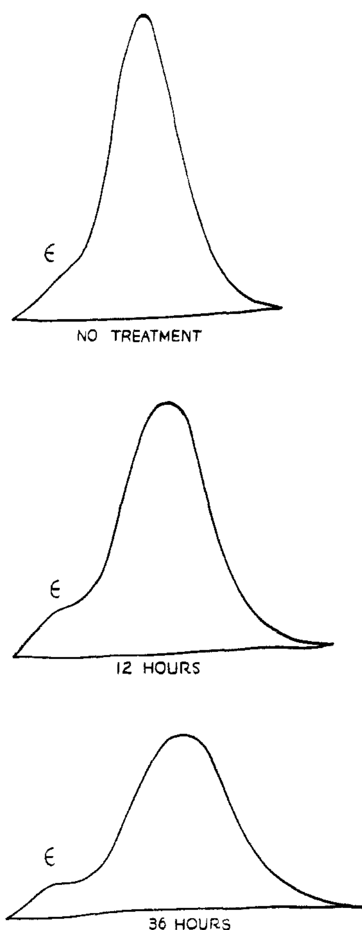

Fig. 2B.—Identical experiments with undigested gamma globulin.

that these increases in mobility are superimposed on increases in electrophoretic heterogeneity of the protein. In this way the determination of the mobility becomes more difficult. The changes are quite similar to the effect of cysteine alone although in these experiments the concentration of cysteine would be rendered relatively low because of the dialysis procedure. The cysteine was added to the globulin system just prior to dialysis. Although these globulins were dialyzed against 0.3% hydrogen peroxide the presence of catalase in the gamma globulins[3,5] undoubtedly rendered the peroxide concentration within the dialysis bag extremely low. The marked increase in electrophoretic mobility of the digested gamma globulin shown in Fig. 2(C) may be the result of a higher effective concentration of the hydrogen peroxide because of an inhibition of catalase activity by the sodium cyanide which was present. A slight tendency to form an anomaly is seen in some of the electrophoretic patterns of the digested gamma globulin. The potential gradient at which the experiments were carried out, while rather high (10 volts per cm.), did not result in the appearance of an anomally with normal gamma globulin with the simple pepsin digested globulin or with globulin systems treated with chemicals for the longer periods of time.

(5) G. E. Perlmann and F. Lipmann, *Arch. Biochem.*, **7**, 159 (1945).

The sedimentation diagrams for the digested globulin plus cysteine remained unchanged after twenty-four hour dialysis against 0.3% hydrogen peroxide. Treatment with hydrogen peroxide at a final concentration of 1% likewise caused no changes other than the appearance of a slight amount of inhomogenous and rapidly sedimenting material.

Marked decreases in the antibody titers of both digested and undigested gamma globulin containing cysteine result after dialysis of eighteen hours duration against 0.3% hydrogen peroxide as shown in Table I. No immunological work was carried out with material treated under more strenuous conditions.

Discussion

An increase in the electrophoretic mobility of serum protein components as a result of the photo-

TABLE I

EFFECT OF CYSTEINE–HYDROGEN PEROXIDE TREATMENT ON IMMUNOLOGICAL TITERS OF HUMAN GAMMA GLOBULIN

	Immunological titers[a]					
	Typhoid agglutinin			PR8 influenza antibody		
Globulin	"H"	"O"	Diphtheria antitoxin	Hirst	Mouse protection	Anticomplementary activity
Digested, control	1.0	0.37	0.8	1.0	0.99	0.01
Digested, treated[b]	0.34	Clouds	.5	1.0	.53	0.01
Undigested, control	.25	0.37	.8	1.0	.98	1.0
Undigested, treated[b]	.06	Clouds	.42	1.0	.42	0.01

[a] Referred to Standard Gamma Globulin A-66 of Enders (J. F. Enders, *J. Clin. Invest.*, **23**, 510 (1944)). [b] Globulins containing 100 mg. of cysteine hydrochloride per g. dialyzed eighteen hours against cold (1°) 0.15 molar sodium chloride containing 0.3% hydrogen peroxide.

oxidation of such systems has been recorded by Tyler and Swingle.[6] It has also been shown that upon denaturation of normal horse serum by heat[7] or by ultraviolet radiation[8] there results a progressive increase in electrophoretic homogeneity leading to the formation of a single component. This peak however possesses the mean mobility of the components initially present and no molecules of higher charge have arisen in the system.

Most of the papain and bromelin digestion products of human gamma globulin were found to possess a marked increase in electrophoretic mobility.[3] Proteolysis should result in the liberation of an equal number of acidic and basic groups, consequently the primary digestion products of gamma globulin of one-half and one-quarter the original molecular weights, would be expected to have essentially the same electrophoretic mobilities as the parent molecule. Such is true in the case of pepsin digested gamma globulin.[2] The electrophoretic behavior of the bromelin and papain digestion products would appear to be due largely to effects other than proteolysis.

The suggestion has been made[3] that the increase in the mobility of gamma globulin or its split products after bromelin digestion may be the result of the reagents used to activate (cyanide, hydrogen sulfide and cysteine) and to inactivate (hydrogen peroxide) the enzyme. The use of such enzyme activating and inactivating agents which result in definite changes in the bromelin molecule as indicated by enzyme activity may likewise cause profound changes in the gamma globulin molecule. The treatment of pepsin digested gamma globulin systems with these chemicals gave rise to an increase in electrophoretic mobility which was associated with marked increase in heterogeneity but not in resolution. Thus, in contrast to systems digested with papain and bromelin no distinct and separate electrophoretic components appeared after oxidation of the pepsin digested gamma globulin. This fact may be understood as an additional indication that proteolytic cleavage by these enzymes results in the production of markedly different molecular fragments. The sedimentation velocity and diffusion data of Petermann[3] and of Bridgman[9] are highly suggestive on this point.

The actual effect of the cysteine and hydrogen peroxide would appear to be the oxidation of some relatively neutral group in the protein molecule to an acid group. A possible effect would be oxidation of cysteine molecules in peptide combination to cysteic acid. This compound has been isolated from human hair oxidized with permanganate[10] and normally occurs in the outer part of the sheep's fleece where the wool is exposed to light and weathering conditions.[11] Its development in any protein or fragment thereof would tend to increase the electrophoretic mobility of the protein. Simple proteolysis could not be expected to result in the production of fragments possessing a markedly lower iso-electric point and hence changes in electrophoretic mobilities at a given *p*H as contrasted with the parent substance. The effect noted with cysteine alone is difficult to rationalize.

The peptic digestion of human gamma globulin does not appear to lower antibody titers.[2] The decrease noted upon digestion of this protein with bromelin and papain[3] may be likewise due to the effects of the cysteine and hydrogen peroxide rather than to proteolysis. The decrease in certain of the antibody titers as shown in Table I resulting from cysteine–hydrogen peroxide treatment of pepsin digested material is to be noted in this connection. Such decrease might be due to the development of non-precipitating or non-agglutinating antibody as occurs in the photo-oxidized systems used by Tyler.[12,13] However, Boyd[14] found that while such treatment (photo-oxidation) destroyed the agglutinating activity of isohemagglutinating sera it did not seem to convert any of the agglutinins into inhibiting ("incomplete" or "blocking") antibody. Our material which has been treated with cysteine–hydrogen peroxide has not been subjected to such a study. However, it is interesting to note that there is a marked difference in the influenza-A antibody titers as assayed by *in vivo* mouse protection and the *in vitro* Hirst tests.

Acknowledgments.—The author is indebted to Dr. J. F. Enders and Miss J. C. Sullivan of the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass., for the immunological assay data reported in this work.

(6) A. Tyler and S. M. Swingle, *J. Immunol.*, **51**, 339 (1945).

(7) J. Vander Scheer, R. W. G. Wyckoff and F. L. Clarke, *ibid.*, **40**, 39 (1941).

(8) B. D. Davis, A. Hollaender and J. P. Greenstein, *J. Biol. Chem.*, **146**, 663 (1942).

(9) W. B. Bridgman, THIS JOURNAL, **68**, 857 (1946).

(10) T. Lissizin, *Z. physiol. Chem.*, **173**, 309 (1928).

(11) A. J. P. Martin and R. L. M. Synge, "Advances in Protein Chemistry," Vol. II, Academic Press Inc., New York, N. Y., 1945, p. 7.

(12) A. Tyler, *J. Immunol.*, **51**, 157 (1945).

(13) *Ibid.*, 329 (1945).

(14) W. C. Boyd, *J. Exp. Med.*, **83**, 221 (1946).

Summary

The electrophoretic mobility and heterogeneity of normal and digested human gamma globulin are increased by cysteine-hydrogen peroxide treatment. It is suggested that such mobility increases result from oxidative changes in the protein. Such treatment likewise causes a marked decrease in the antibody titers of human gamma globulin.

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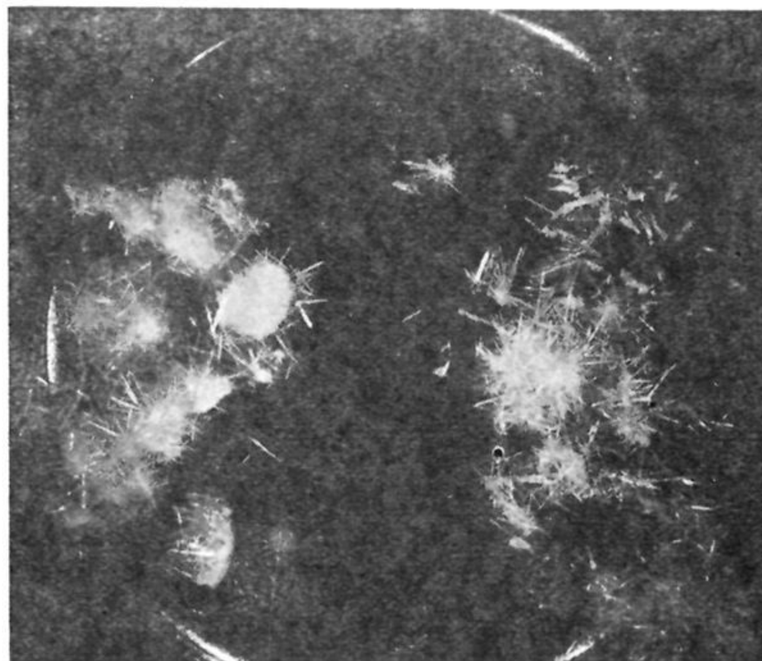
Esters of α-Aminoisobutyric Acid

BY RALPH A. JACOBSON

Although the methyl and ethyl esters of the more common α-amino acids have been described, in many instances the corresponding esters of the higher alcohols are unknown. The work described in the present paper was undertaken in order to obtain information regarding the properties and, in particular, the stability toward cyclization of the higher esters of α-aminoisobutyric acid.

The preparation of α-aminoisobutyric acid in 30–33% yields by the hydrolysis of α-aminoisobutyronitrile with hydrobromic acid is described in "Organic Syntheses."[1,2] In the present study, 70–75% yields of the acid were obtained by the following two procedures: (a) the hydrolysis of α-aminoisobutyronitrile with 40% sulfuric acid according to the general procedure suggested by Cocker and Lapworth[3] and (b) the hydrolysis of 5,5-dimethylhydantoin with 60% sulfuric acid according to the procedure of Bucherer and Steiner.[4] Procedure (b) is to be recommended since 5,5-dimethylhydantoin is easily prepared in excellent yield.[5]



A B

Fig. 1.—A = condensation product from dodecyl ester of α-aminoisobutyric acid; B = tetramethyldiketopiperazine.

The isobutyl, octyl and dodecyl esters of α-aminoisobutyric acid were prepared by esterification in the presence of hydrogen chloride.[6] The hydrochlorides of the esters were first isolated for characterization and then converted to the free esters by treatment with alkali. The hydrochlorides of the esters are crystalline solids and the free esters are colorless liquids of pronounced basicity. The isobutyl ester is slightly soluble in water but the octyl and the dodecyl esters are practically insoluble. The hydrochlorides of the isobutyl and octyl esters are soluble but the dodecyl ester hydrochloride is insoluble in water.

In general, the methyl and ethyl esters of α-amino acids tend to cyclize to diketopiperazine derivatives or to form condensation products even at room temperature. The lower alcohol esters of glycine are particularly unstable and readily form glycine peptide ester chains of considerable length.[7] The methyl ester of α-aminoisobutyric acid, ($NH_2C(CH_3)_2COOCH_3$), appears to be somewhat more stable possibly because of the doubly substituted alpha carbon. Thus Franchimont and Friedmann[6] obtained tetramethyldiketopiperazine in only 34% yield even after prolonged heating of the ester at 230–240°. The present results indicate that the higher esters of α-aminoisobutyric acid possess still greater stability. At room temperature, samples of the isobutyl, octyl and dodecyl esters formed only a trace of solid material over a period of many months. At 210–220° the octyl ester yielded 3.0% of condensation product after twenty-two hours. At its boiling point (177–179°) the isobutyl ester yielded only 0.91% of

(1) Clarke and Beane, "Organic Syntheses," **XI**, 4 (1931).

(2) Lin and Li, *J. Chinese Chem. Soc.*, **6**, 88 (1938); *C. A.*, **35**, 5096 (1941), prepared the acid by a circuitous route.

(3) Cocker and Lapworth, *J. Chem. Soc.*, 1391 (1931).

(4) Bucherer and Steiner, *J. prakt. Chem.*, **140**, 291 (1934).

(5) (a) Ref. 4; (b) Wagner and Baizer, "Organic Syntheses," **20**, 42 (1940).

(6) Methyl α-aminoisobutyrate has been prepared by Franchimont and Friedmann, *Rec. trav. chim.*, **27**, 201 (1908), and ethyl α-aminoisobutyrate by Zelinski and Kulikow, *Z. physiol. Chem.*, **73** 459 (1911); *Chem. Zentr.*, **82**, 1319 (1911). The bactericidal properties of the hydrochloride of the dodecyl ester have been examined by Baker, Harrison and Miller, *J. Exptl. Med.*, **74**, 611 (1941).

(7) (a) Frankel and Katchalski, THIS JOURNAL, **64**, 2264 (1942); **64**, 2268 (1942). (b) Pacsu, *Nature*, **144**, 551 (1939); Pacsu and Wilson, *J. Org. Chem.*, **7**, 117 (1942); **7**, 126 (1942).