D. J., Mercer, G. A., and Wright, H. J. (1961), *J. Chem. Soc.*, 1183.
Badenhuizen, N. P. (1959), *Protoplasmatologia 2*, 58.
Bailey, J. M., and Whelan, W. J. (1961), *J. Biol. Chem. 236*, 969.
Barker, S. A., Bourne, E. J., and Peat, S. (1949), *J. Chem. Soc.*, 1705.
Barker, S. A., Bourne, E. J., Peat, S., and Wilkinson, I. A. (1950), *J. Chem. Soc.*, 3022.
Bernier, G. M., and Putman, F. W. (1963), *Nature 200*, 223.
Blatt, F. W., Feinberg, M. P., Hoffenberg, H. B., and Saravis, C. A. (1965), *Science 150*, 224.
Bourne, E. J., and Peat, S. (1945), *J. Chem. Soc.*, 877.
Brakke, M. K. (1963), *Anal. Biochem. 5*, 271.
Cleland, W. W. (1964), *Biochemistry 3*, 480.
Dygert, S., Li, L. H., Florida, D., and Thoma, J. A. (1965), *Anal. Biochem. 13*, 367.
Edsall, J. T. (1953), *Proteins IB*, 634.
Geddes, R., and Greenwood, C. T. (1969), *Staerke 21*, 148.
Griffin, H. L., and Wu, Y. V. (1968), *Biochemistry 7*, 3063.
Gunja, Z. H., Manners, D. J., and Maung, K. (1960), *Biochem. J. 75*, 441.
Hobson, P. N., Whelan, W. J., and Peat, S. (1950), *J. Chem. Soc.*, 3566.
Igaue, I. (1963), *Agr. Biol. Chem. 26*, 424.
Manners, D. J. (1962), *Advan. Carbohyd. Chem. 17*, 373.
Manners, D. J., Rowe, J. J. M., and Rowe, K. L. (1968), *Carbohyd. Res. 8*, 72.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Peat, S., Whelan, W. J., and Bailey, J. M. (1953), *J. Chem. Soc.*, 1422.
Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys. Acta 112*, 346.

Reversible Inactivation of the Potential Pepsin Activity of Pepsinogen by Alcohols*

Hava Neumann† and Meir Shinitzky

ABSTRACT: The effect of a series of aliphatic alcohols, extending from methanol to 1-hexanol, on the native conformation of pepsinogen was investigated by enzymatic and spectral methods. It was found that the presence of an alcohol exerts on pepsinogen a specific conformational change by which it is transformed to a form which is insusceptible to acid treatment under which native pepsinogen is fully converted to pepsin. This was demonstrated by the absence or partial loss of the potential pepsin activity of pepsinogen upon alcohol treatment. The effectiveness of the alcohols on the above transformation was found to increase with chain length and concentration of the alcohol, as determined at pH 8.0 and 35°, and to depend on pH and temperature. Thus, the molarity of the alcohol which causes 50% reduction in the potential pepsin activity of pepsinogen, $M_{1/2}$, is 2.67 for methanol and only 0.024 for 1-hexanol. The alcoholic inactivation is fully reversible and by dialysis or dilution with the employed buffer the potential pepsin activity of pepsinogen can be fully recovered. Moreover, when alcohol-denatured pepsinogen is exposed to acid (pH 1.7 for 2 min), and then transferred to neutral pH, a full regain in its potential pepsin activity is achieved after 1 hr. Alcoholic solutions of pepsinogen display changes in specific optical rotation at 366 nm, $[\alpha]_{366}$, and in CD, absorption, and fluorescence spectra. The changes in $[\alpha]_{366}$ and in CD spectrum which follow ethanolic treatment of pepsinogen were studied in detail, and were found to be reversible and parallel to the apparent change in potential pepsin activity of pepsinogen. Ethanol-treated pepsinogen also shows slower rate of diffusion in immunodiffusion experiments. Based on all the above observations, a kinetic mechanism for the effect of alcohols on pepsinogen is presented. According to this mechanism when n molecules of alcohol are bound to pepsinogen it can be transformed to an acid inactivable form. The values of n were found to be 6 for methanol and ethanol, and 4 for 1-butanol. This study leads to the conclusion that the postulated intramolecular cleavage of the acid-labile bond during the process of activation of pepsinogen requires a definite conformation of the zymogen. It is speculated that this conformation in acid is of high strain energy which is exploited as energy of activation in the pepsinogen-to-pepsin conversion.

At the pH region of 6.5–8.5 and at temperature below 40° pepsinogen is stable and retains full potential pepsin activity (Perlmann, 1963, 1967, 1970; Grizzuti and Perlmann, 1969). At acidic solutions pepsinogen is converted to pepsin whereas above pH 9 its potential pepsin activity is markedly reduced (Ryle, 1970). Inactivation of pepsinogen by a short exposure to pH 10–11 is fully reversible; activity can be recovered by reducing the pH back to neutral. However, alkaline exposure of more than several minutes will cause an irreversible loss of potential pepsin activity of pepsinogen, the extent of which increases with time of exposure. Similarly, heating of a neutral solution of pepsinogen up to 60° is followed by a complete deactivation of the zymogen, yet if the pepsinogen solution is kept at this temperature for only several minutes and then cooled to 25° the potential pepsin activity is completely restored. A series of heating and cooling, however,

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will gradually decrease the potential pepsin activity of pepsinogen. Urea denaturation of pepsinogen is also found reversible and upon dialysis of the urea the potential pepsin activity and part of the optical polarity are recovered (Perlmann, 1963, 1967). In the preceding study we demonstrated that under the conditions at which pepsinogen is stable, the presence of aliphatic alcohols reversibly denature the protein to a form which is particularly characterized by being insusceptible to conversion to pepsin by acid. It is thus concluded that the formation of pepsin by intramolecular cleavage of pepsinogen in acid requires a unique conformation of the zymogen.

Experimental Procedure

Materials. Two types of pepsinogen, one crystallized by the method of Herriott (1938), Worthington Lot PG OFA, and the other of Worthington Lot PGC 9BA, were chromatographed on DEAE-Sephadex according to Rajagopolan *et al.* (1966) before use. All the alcohols used were of spectroscopic grade. Hemoglobin substrate powder was also purchased from Worthington. All other chemicals were of analytical or reagent grade from commercial sources.

Buffers. Phosphate buffer solutions of 0.1 M were prepared according to Cohn (1927), using stock solutions of 0.2 M sodium dihydrophosphate and 4.0 M sodium hydroxide. Appropriate amounts of the sodium hydroxide solution were introduced into the sodium dihydrophosphate solution, and then the proper amounts of conductivity water were added to achieve a 0.1 M phosphate concentration.

Enzyme Assays. Activation of pepsinogen was accomplished by adding the pepsinogen or pepsinogen–alcohol preparation to the hemoglobin substrate at pH 1.7, according to Tanksley *et al.* (1970). Assays for pepsin activity were conducted by the method of Anson (1948) as modified by Ryle and Porter (1959). In most assays, 10–25 μl of the reaction mixture was added to 1.0 ml of 2% acid-denatured hemoglobin solution, pH 1.7. After 10-min incubation at 35°, the reaction was terminated by addition of 5 ml of 5% (w/v) trichloroacetic acid. The insoluble residue was removed by filtration through Whatman No. 3 paper, and the pepsin activity was determined by measuring the absorbance of the trichloroacetic acid soluble degradation products in the filtrate at 280 mμ against the appropriate blank. Assays were performed in triplicate or quadruplicate because of the 5% deviations in the reproducibility of the hemoglobin test. In order to exclude the possibility that the reduction in the potential pepsin activity of pepsinogen is due to direct inhibition of pepsin by the various alcohols (Tang, 1965) pepsin activity was measured in separate assays in the presence of the highest final concentration of each of the alcohols used (0.2% for methanol to 0.01% for 1-hexanol).

Alcohol–Pepsinogen Preparations. Unless otherwise specified, pepsinogen was dissolved in 0.1 M sodium phosphate buffer, pH 8.0, and cooled in an ice bath. The appropriate amount of alcohol was then added slowly from a microsyringe in order to minimize the immediate denaturation of the zymogen. The pepsinogen concentration in most experiments was 1 mg/ml $\sim 2 \times 10^{-5}$ M. The preparations were incubated for the desired period of time in the presence of various concentrations of the alcohols investigated. In some experiments in 10% ethanol the pH value of the buffer was changed at the region of pH 7–9, at a constant temperature of 35°, while in another set of experiments the temperature of the incubation was changed between 25 and 40° at a constant pH of 8.0. In all the control experiments the alcohol was omitted.

Optical Rotation and Circular Dichroism Measurements. Optical rotation (OR) at 366 nm and circular dichroism (CD) measurements were made in a Cary Model 60 recording spectropolarimeter equipped with a Cary Model 6002 circular dichroism attachment. The OR data are reported as specific optical rotation, $[\alpha]_{366} = [\alpha/cl][3/(n_{366}^2 + 2)]$, and the CD data as the mean residue ellipticity, $[\theta]_\lambda = [\theta_\lambda(M_0/cl)]$, where α and θ_λ are the measured rotation and ellipticities at the wavelength λ, M_0 is the mean residue molecular weight, taken as $M_0 = 111$ according to the amino acid composition of pepsinogen given by Arnon and Perlmann (1963), c is the protein concentration in grams per 100 ml, l is the path length of the cell used in decimeters, and n_λ is the refractive index of the solvent at the wavelength λ. Since the indexes of refraction of most of the solvents used differ from that of water, the specific rotation $[\alpha]$ and the mean residue ellipticity $[\theta]$ were corrected for the corresponding values in water with the aid of the equation (Foss and Schellman, 1964; Davidson and Fasman, 1967)

$$[\alpha]_w^{25} \text{ corrected} = [\alpha]_0^{25} \frac{n_w^2 + 2}{n_s^2 + 2} \quad \text{(I)}$$

where n_w is the index of refraction of water and n_s is the index of refraction of the solvent both for $\lambda = 589$ nm. The variation of the index of refraction with wavelength was estimated by using the Sellmeier equation (Fasman, 1962)

$$n^2 = 1 + \frac{A\lambda^2}{\lambda^2 - \lambda_0^2} \quad \text{(II)}$$

where A and λ_0 are constants which were derived from two simultaneous equations for known values of n_λ (International Critical Tables, 1930).

Fused quartz cells with 1.0-cm light path were used with protein concentration of 0.1–0.5%. Water from an external bath was circulated through jacketed cells to control the temperature to ±0.02°. All measurements were made after equilibration at each temperature for 10 min, or until an invariant measured value was obtained.

Other Procedures. All measurements of pH were made with a Radiometer Model TTTlc titrator, with G202B electrodes. Protein concentrations of pepsinogen were calculated from the absorbance at 278 mμ employing a molar extinction coefficient of 5.17×10^4 (Arnon and Perlmann, 1963). Optical density measurements were made with a Zeiss PMQ II spectrophotometer. Difference spectra measurements were made in tandem of two sets of matched 1-cm path length cells, with a Cary Model 14 recording spectrophotometer.

Results

Effect of Water-Miscible Aliphatic Alcohols on the Potential Pepsin Activity of Pepsinogen. Inasmuch as alcohols in low concentration were found not to inhibit pepsin (see Experimental Section) it was possible to study the extent of denaturation of pepsinogen by various alcohols by using the potential pepsin activity of pepsinogen, as a measure of the extent of reaction of alcohols with pepsinogen. Preliminary experiments indicated that the most convenient conditions for the study of the effects of different alcohols was in 0.1 M sodium phosphate buffer, pH 8.0, at 35°. A solution of 0.5 mg/ml of pepsinogen in 0.1 M sodium phosphate buffer, pH 8.0, was incubated at 35° in the presence of an alcohol from the series listed in Table I, at various concentrations. After 10 min of incuba-

TABLE I: The Molarity ($M_{1/2}$) and Percentage of Various Aliphatic Alcohols Which Cause 50% Reduction in Potential Pepsin Activity of Pepsinogen. The Data Were Taken from Figure 1.

Alcohol	% v/v	$M_{1/2}$
Methanol	10.4	2.67
Ethanol	8.0	1.37
2-Propanol	5.1	0.66
1-Propanol	3.5	0.46
1-Butanol	1.6	0.175
1-Pentanol	0.8	0.074
1-Hexanol	0.3	0.024

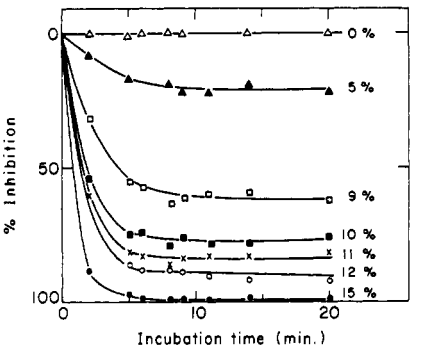

FIGURE 2: The change in percentage inactivation of potential pepsin activity of pepsinogen by different amounts of ethanol with time of incubation in 0.1 M sodium phosphate buffer, pH 8.0 at 35°.

tion an aliquot of the alcoholic mixture was introduced into acidic hemoglobin solution for pepsin activity measurement (see Experimental Section). The results of these experiments are summarized in Figure 1. As indicated in Figure 1, the effectiveness of the alcohols in reducing the potential pepsin activity of pepsinogen increases with chain length. This suggests that hydrophobic interaction between the hydrocarbon chain of the alcohol and the zymogen plays a major role in the inactivation. The molarities of the alcohols which exert 50% reduction of the pepsin activity, $M_{1/2}$, derived from Figure 1, are given in Table I.

The time course of the alcohol inactivation of pepsinogen was determined in the above mixtures by measuring the pepsin activity at minute time intervals after introducing the alcohol. A rapid loss of potential activity, strongly dependent on alcohol concentrations, was observed. In all concentrations studied an equilibrium stage was always reached after 10 min. The time course curves of loss of potential pepsin activity in the presence of various concentrations of ethanol and 1-butanol are shown in Figures 2 and 3.

Effect of pH on the Ethanol–Pepsinogen Reaction. The effect of pH on the alcohol inactivation of pepsinogen was studied with ethanol (10%, v/v) at 35°. The per cent inhibition was determined after 20 min of incubation at each pH, and the results are given in Figure 4. A sharp increase in the reduction of potential activity occurred between the apparent pH of 7.8 and 8.4, the plot of which resembles a titration curve. This pH dependence could result either from a direct reaction of the alcohol with the unprotonated state of a residue in the protein having a pK_a value around 8, or from a conformational change in pepsinogen which increases with pH in the presence of 10% ethanol. Conformational changes normally occur at a narrow temperature range, whereas changes in ionization state are less temperature dependent; therefore, it appeared that a study of the temperature dependence of the reaction may reveal which of the two possibilities prevails in this case.

Effect of Temperature on the Ethanol–Pepsinogen Reaction. The temperature dependence of the change of potential pepsin activity of pepsinogen at various temperatures in the presence of 10% ethanol at pH 8.0 was studied enzymatically (see Experimental Section). The reduction of the potential activity was found to be very rapid, but at each temperature the equilibrium stage was reached. The per cent inhibition of the potential pepsin activity of pepsinogen at each equilibrium stage was plotted *vs.* temperature, as given in Figure 5.

The sharp temperature dependence of the reaction suggests that it is linked to a conformational change of the protein. This possibility and the mutual dependence of the two events (loss of potential pepsin activity of pepsinogen and the conformational change) were further investigated by optical methods.

Dependence of the Specific Optical Rotation $[\alpha]_{366}$ of Pepsinogen on Ethanol Concentration. Measurements of $[\alpha]_{366}$ were carried out with 3 mg/ml solution of pepsinogen in 0.1 M sodium phosphate buffer, pH 8.0, in the presence of various

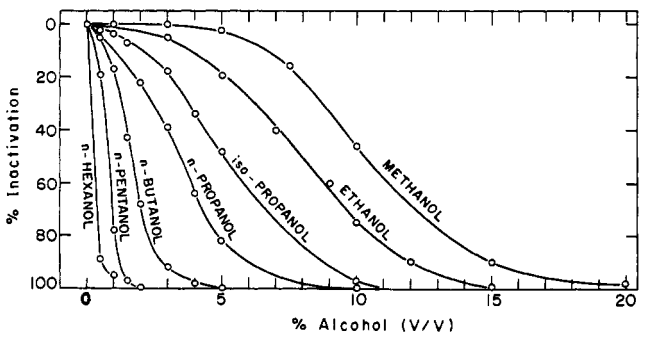

FIGURE 1: Percentage inactivation of potential pepsin activity of pepsinogen after 10 min of incubation with different amounts of various alcohols in 0.1 M sodium phosphate buffer, pH 8.0 at 35°.

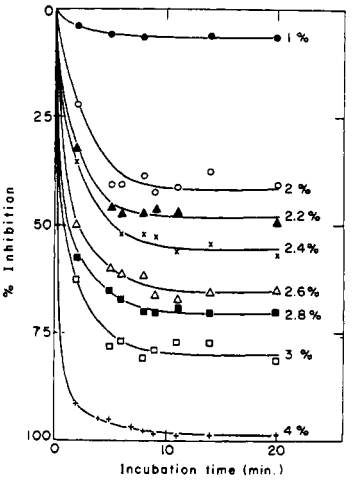

FIGURE 3: The change in percentage inactivation of potential pepsin activity of pepsinogen by different amounts of 1-butanol with time of incubation in 0.1 M sodium phosphate buffer, pH 8.0 at 35°.

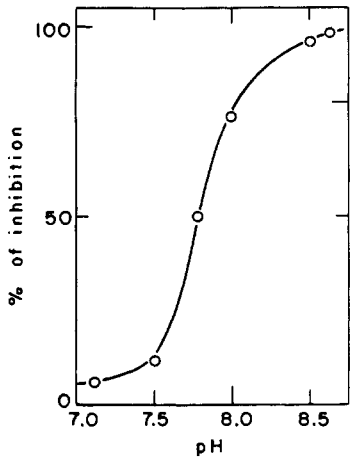

FIGURE 4: The apparent pH profile of percentage inactivation of potential pepsin activity of pepsinogen by 10% ethanol at 35°. Measurements were performed after 10–30 min of incubation.

amounts of ethanol. For each system a series of values for $[\alpha]_{366}$ at different temperatures in the range 10–70° were recorded.

As illustrated in Figure 6, the specific rotation $[\alpha]_{366}$ of pepsinogen in the presence of 10% ethanol decreases from −203 to −235° with increasing temperature with a transition midpoint of 31°. The transition midpoints were found to decrease with increasing concentration of ethanol. For alcohol-free solution the transition midpoint obtained was 43° which is consistent with the value of 47.5° at pH 7.7 reported by Grizzuti and Perlmann (1969). The other transition midpoints obtained were 36° for 5% ethanol and 26.5° for 15% ethanol. All transition midpoints found practically coincide with the 50% loss of potential pepsin activity of pepsinogen checked by enzymatic assays. Furthermore, each point of the transition curve of 10% ethanol fits well with the per cent of reduction of potential pepsin activity of pepsinogen (see Figure 5).

Effect of Alcohols on the Ultraviolet Spectral Properties of Pepsinogen. The CD, absorption difference, and fluorescence spectra of pepsinogen were measured in alcoholic and alcohol-free solutions. Solutions of 1 mg/ml of pepsinogen in 0.1 M sodium phosphate buffer, pH 8.0, with 0, 5, 10, and 15% ethanol were incubated at 35° for 1 hr before measurements of CD spectra. The CD spectra were recorded at 29° in the tyrosine and tryptophan absorption region of 250–310 nm. The

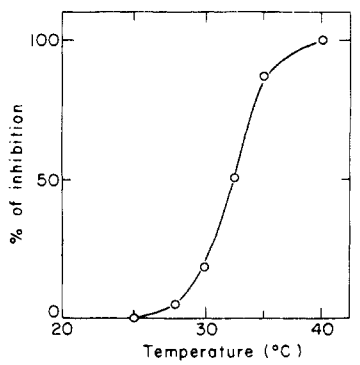

FIGURE 5: The temperature dependence of percentage inactivation of potential pepsin activity of pepsinogen by 10% ethanol in 0.1 M sodium phosphate buffer, pH 8.0. Measurements were performed after 30 min of incubation.

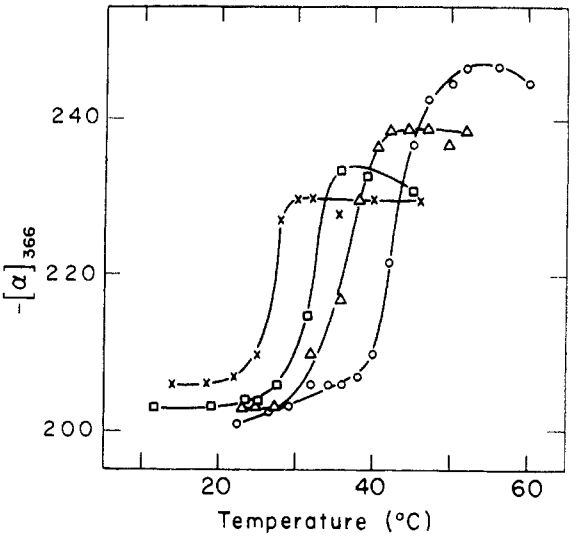

FIGURE 6: The temperature profile of the specific optical rotation of pepsinogen at 366 nm, $[\alpha]_{366}$, in the presence of various amounts of ethanol in 0.1 M sodium phosphate buffer, pH 8.0. (O—O) No ethanol; (Δ—Δ) 5% ethanol; (□—□) 10% ethanol; (×—×) 15% ethanol.

effect of ethanol on the CD spectrum of native pepsinogen is characterized by the disappearance of a small negative dichroic band at 280 nm as demonstrated in Figure 7. When the CD spectra given in Figure 7 were recorded immediately after the addition of ethanol, the observed effect of the alcohol was found to be considerably smaller.

Difference spectra of pepsinogen before and after alcohol treatment were recorded with 0.4 mg/ml of protein in 0.1 M sodium phosphate buffer, pH 8, at 35° 1 hr after introducing the alcohol. Figure 8 shows the difference spectra obtained with 10% ethanol, 4% 1-butanol, and 0.5% 1-hexanol. The figure indicates a blue shift in the total absorption spectra of the tyrosines and the tryptophans due to binding of the

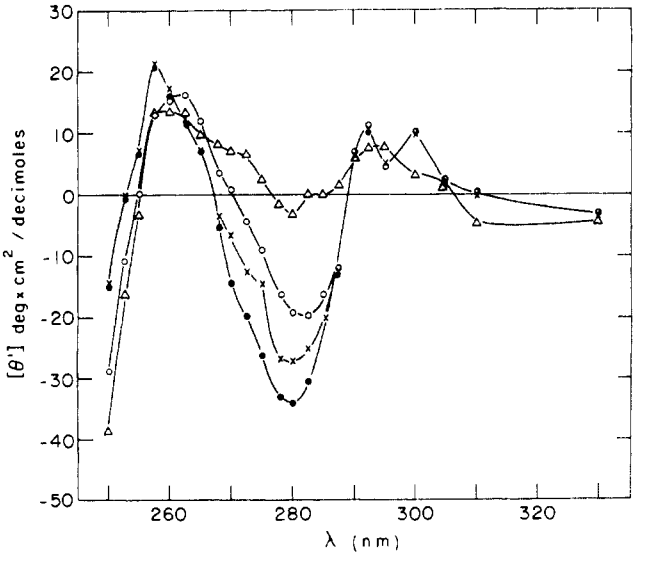

FIGURE 7: Corrected circular dichroism spectra of pepsinogen in 0.1 M sodium phosphate buffer, pH 8.0 at 29°, in an alcohol-free solution (●—●), and in the presence of 5% ethanol (×—×), 10% ethanol (O—O), and 15% ethanol (Δ—Δ). Spectra were recorded after 1 hr of incubation at 35°.

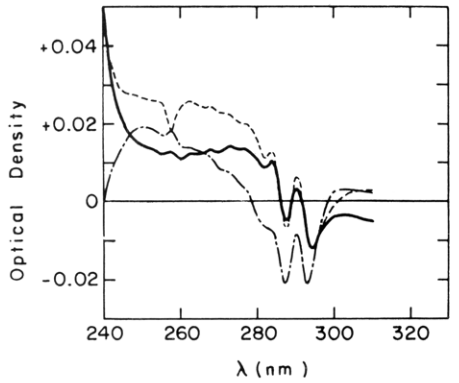

FIGURE 8: Difference spectra of pepsinogen (OD_{082}^{1cm} = 0.760) in 0.1 M sodium phosphate buffer, pH 8.0 at 35°, in the presence of 10% ethanol (·—·), 4% 1-butanol (– – –), and 0.5% 1-hexanol (——). Spectra were recorded at a tandem of two matched 1-cm cuvets. The measured system contained the alcoholic pepsinogen solution in one cuvet and buffer in the other, whereas the reference system contained pepsinogen in buffer in one cuvet and the appropriate alcohol–buffer mixture in the other. Measurements were taken after 1 hr of incubation.

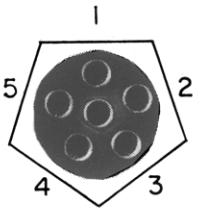

FIGURE 9: Immunodiffusion in agar gel. Center well—antipepsinogen. Other wells: 1—native pepsinogen (A_1); 2—pepsinogen incubated for 30 min at pH 8.0 and 35° in the presence of 15% ethanol (B); 3—pepsinogen inactivated with 15% ethanol, exposed to pH 1.7 for 2 min, and then diluted tenfold with 0.1 M sodium phosphate buffer, pH 8.0 (A_3); 4—pepsinogen inactivated with 15% ethanol and then diluted tenfold with 0.1 M sodium phosphate buffer, pH 8.0 (A_2); 5—native pepsinogen exposed to pH 1.7 for 2 min and then diluted with 0.1 M phosphate buffer, pH 8.0 (DP). In all experiments the final concentration of pepsinogen was 1 mg/ml.

alcohols by the protein. It is worthwhile noting that similar difference spectra were found with α-chymotrypsinogen at pH 2.8 in the presence of high concentrations of alcohols (Herskovitz *et al.*, 1970).

Fluorescence spectra were measured with 0.1 mg/ml of pepsinogen in 0.1 M sodium phosphate buffer, pH 8.0, in the presence of 15% ethanol and in alcohol-free solutions. Both systems display an emission maximum at 341 nm; however, the fluorescence intensity of pepsinogen in the alcoholic solution is enhanced by 22%.

The spectral changes described reflect alteration in the intrinsic environment around some of the tyrosines or the tryptophans upon alcohol binding to pepsinogen. The blue shift in absorption spectra in addition to the fluorescence enhancement suggest that the alcohol binding unfolds regions of the protein which mainly expose tryptophans to interaction with the side chains of the alcohols. A reversible decrease of fluorescence efficiency upon urea or temperature denaturation of pepsinogen was reported by Perlmann (1964).

Immunological Tests. The homologous and heterologous immunological interaction of antibodies to pepsinogen were carried out in agar gel diffusion according to Ouchterlony (1948), with the following antigens: native pepsinogen (A_1); ethanol-denatured pepsinogen (B); ethanol-denatured pepsinogen reactivated by dilution of 0.1 M sodium phosphate buffer, pH 8 (A_2); ethanol-denatured pepsinogen treated by acid and diluted with 0.1 M sodium phosphate buffer, pH 8.0 (A_3); and native pepsinogen treated by acid and diluted with 0.1 M sodium phosphate buffer, pH 8.0 (DP). The final concentration of the protein in each sample was 1.0 mg/ml. Prior to the immunological tests, enzymatic assays were performed on each sample to be sure that enough time was given to regain the equilibrium state in each case and in order to compare the enzymatic and immunological behavior of the samples. In this test the activities, measured by hemoglobin assay (see Experimental Section) of A_1, A_2, and A_3, were identical while sample B or DP had no or only a small amount of activity.

The immunological interactions of the different pepsinogen preparations quoted above with the antipepsinogen serum are shown in Figure 9. As is apparent from this figure, A_2 and A_3 reacted with the antibodies as the native pepsinogen (A_1). However, the native pepsinogen treated with acid and diluted by base (DP) missed determinant(s) present in the antibodies. Sample B—the alcohol-denatured pepsinogen—gave with the antibodies an additional "slower" moving band, which is likely to be due to the unfolded structure of the alcohol-denatured pepsinogen.

Reversibility of the Alcoholic Inactivation of Pepsinogen. The potential pepsin activity of alcohol-denatured pepsinogen was tested after dilution with buffer and after dialysis of the alcohol; 1 mg/ml of pepsinogen in 0.1 M sodium phosphate buffer, pH 8.0, at 35° was incubated for 20 min with various amounts of ethanol. The mixture was then diluted tenfold with the employed buffer and aliquots of 0.1 ml were taken at time intervals for the pepsin assay. The determined pepsin activity was compared to that obtained with parallel alcohol-free pepsinogen solution, and in all cases practically full recovery of activation was found 3 hr after the dilution. The reactivation curves obtained for 10 and 15% ethanol are shown in Figure 10. Similarly, upon exposing the alcoholic pepsinogen solution to dialysis the activity is slowly regained and reaches that of the intact zymogen after the complete removal of the alcohol. In addition to that, as demonstrated in Figure 11, by dialysis the CD spectrum of pepsinogen in alcoholic solution returns to that of pepsinogen in alcohol-free solution.

Resistance of the Alcohol-Denatured Pepsinogen to Acid. Pepsinogen (1 mg/ml) in 0.1 M sodium phosphate buffer, pH 8.0, in the presence of 15% ethanol and in alcohol-free solution was incubated at 35° for 20 min. The potential pepsin activity of the alcoholic pepsinogen mixture was found to be, as in the previous experiments, only 2% of that of the alcohol-free solution; 20 μl/ml of 0.3 M HCl was then added to the two solutions to reduce the pH to 1.7. After 2 min of exposure to acid the solutions were diluted tenfold with 0.1 M sodium phosphate buffer, pH 8.0, at 35° which increased the pH to 7.8. Aliquots (0.1 ml) of the final solutions were taken at minute time intervals after the dilution for pepsin activity assay, and the results were compared with those obtained with alcohol intact pepsinogen. Pepsin activity was found to gradually recover and full recovery reached 60 min after neutralization. Since pepsin undergoes an irreversible denaturation at pH < 6 (Ryle, 1970) the residual pepsin activity observed after neutralization of the acidic solution is only due to pepsinogen which was not converted to pepsin because of the alcohol treatment. The practical full reactivation thus indi-

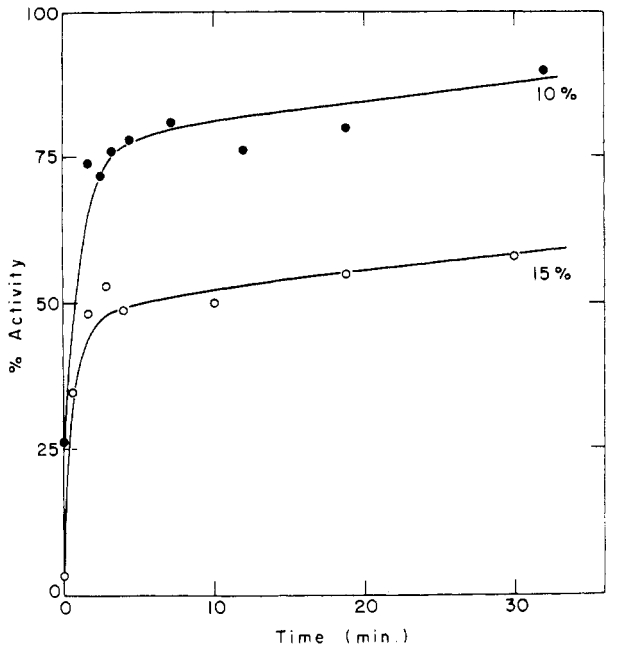

FIGURE 10: Time course of reactivation of pepsinogen inactivated by 10 and 15% ethanol in 0.1 M sodium phosphate buffer, pH 8.0, which followed by tenfold dilution with the employed buffer.

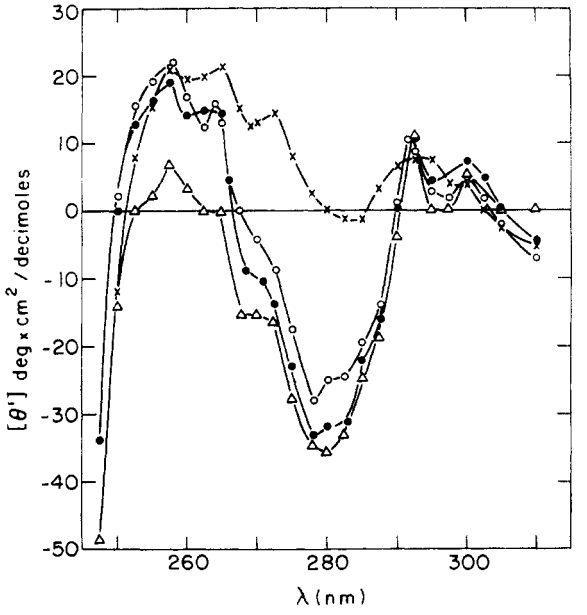

FIGURE 11: Corrected circular dichroism spectra of pepsinogen in 0.1 M sodium phosphate buffer, pH 8.0 and 29°. (●—●) In alcohol-free solution; (×—×) in the presence of 15% ethanol; (○—○) after half an hour of dialysis; (Δ—Δ) after 3 hr of dialysis against 2 l. of the employed buffer.

cates that the alcohol-treated pepsinogen is transformed into an acid-resistant form which returns to natural pepsinogen at neutral pH.

Discussion

This communication deals with the finding that water-miscible alcohols inactivate the potential pepsin activity of pepsinogen. It is shown that the inactivation ability increases with chain length of the alcohol, and for the two extremes in the tested series, 1-hexanol was found to be about a hundred times more effective than methanol (see Figure 1 and Table I). As shown in Figures 4 and 5 the presence of alcohol decreases the denaturation transition temperature or pH proportionally to its amount. This implies that the alcohol-denatured form is identical with that which is obtained in an alcohol-free solution at pH > 10 or at temperatures above 50° (Perlmann, 1963, 1967), and that the transformation of the pepsinogen-alcohol complex to the denatured form occurs more readily than that of the intact protein. The changes in CD, absorption, and fluorescence spectra which follow the alcohol inactivation of pepsinogen indicate conformational changes which expose some aromatic residues, probably tryptophans, to interaction with the bound alcohol molecules. The assumed conformational change is further supported by the observation of difference in the rate of diffusion between the active and the alcohol-inactivated pepsinogen in the immunodiffusion experiments.

By dilution or dialysis of inactivated pepsinogen in ethanolic mixture the potential pepsin activity is essentially fully recovered (see Figure 10). Parallel to the reactivation the CD spectrum returns to that of the active pepsinogen (Figure 11). Thus, the effect of alcohols on the conformation of pepsinogen is completely reversible. Moreover, when an alcohol-inactivated pepsinogen is transferred into acidic solution, in which its active form is rapidly transformed to pepsin, the pepsin activity is markedly reduced. However, when the acidic solution is transferred back to neutral pH, the activity is gradually recovered, and after 60 min it is essentially fully recovered. The inactivation–reactivation cycles can be repeated several times with virtually no loss in potential pepsin activity of pepsinogen.

All the findings presented, together with those reported (Perlmann, 1963, 1967), strongly indicate that pepsinogen can possess two exchangeable forms: an acid-activable form (denoted as A) and an acid-resistant form (denoted as B). At neutral pH values and at temperatures below 50° pepsinogen is in the A form only. Increasing the temperature to about 60° or the pH to 10–11 will result in a complete and reversible transformation to the B form (Perlmann, 1963, 1967). However, under such conditions B is unstable and will gradually and irreversibly denature. Upon the addition of alcohol (E) to pepsinogen in the A form (at neutral pH and temperature below 50°) transition to B takes place. This can be explained by assuming that A forms a nonspecific complex (C) with up to n molecules of alcohol which can dissociate to B but only when it contains n molecules of alcohol. B is then further transformed to A since under the chosen conditions A is the stable form of pepsinogen. This transformation closes the relation between A, B, and C to the following cyclic equilibrium

$$A + nE \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \xrightarrow{k_3} B + nE \qquad (1)$$

$$B \xrightarrow{k_1} A$$

The reversible binding of n molecules of alcohol E to A which forms the complex C is equivalent to a series of n equilibria of A with 1 to n molecules of E in which the binding is highly cooperative. The dissociation of C to B plus alcohol is assumed nonreversible since when alcohol is added to pepsinogen in the B form (at pH 10 or at 60°) no conversion to the activable form A is observed.

Under steady-state conditions, and when the alcohol is in large excess compared to pepsinogen, the following relations prevail

$$[C] = \frac{k_2}{k_{-2}}[A]e^n = K_2[A]e^n$$

$$\frac{d[B]}{dt} = k_3[C] - k_1[B] = 0; \quad [B] = \frac{k_3}{k_1}[C] = \frac{k_3}{k_1}K_2[A]e^n \tag{2}$$

where e is the total molarity of the alcohol. At the above conditions the fraction of the total concentration of pepsinogen, p, which is in the A form is given by

$$\frac{[A]}{p} = \frac{1}{1 + K_2\left(1 + \frac{k_3}{k_1}\right)e^n} \tag{3}$$

When the alcoholic mixture is diluted into acid, A is very rapidly converted to pepsin (Ryle, 1970), B stays unaltered, and C dissociates $k_{-2}/(k_{-2} + k_3)$ parts to A and $k_3/(k_{-2} + k_3)$ parts to B. However, dissociation of C to A should result in increase of the residual activity, and as the presented results show that 100% inactivation of pepsinogen by alcohols is easily reached, it implies that $k_3 \gg k_{-2}$ and that C dissociates virtually only to the B form. Thus, in a good approximation, the fraction of pepsinogen which is in the active form A at the pepsin assay conditions is given by eq 3 and equals the measured fraction of pepsin activity α. Equation 3 can be reset to the following linear relationship

$$\log\left(\frac{1}{\alpha} - 1\right) = \log K' + n \log e$$

where

$$K' = K_2\left(1 + \frac{k_3}{k_1}\right) \tag{4}$$

Figure 12 shows plots of $\log[(1/\alpha) - 1]$ *vs.* $\log e$ for methanol, ethanol, and 1-butanol, data taken from Figures 1, 2, and 3. Straight lines are obtained with corresponding slopes of n = 5.7 for methanol, n = 6.1 for ethanol, and n = 4.2 for 1-butanol, and intercepts of K' = 0.006, 0.1, and 300 for methanol, ethanol, and 1-butanol, respectively. If the suggested kinetic scheme represents the actual kinetic events, and is not a simplified equivalent scheme, these figures suggest that under the chosen conditions when six molecules of methanol or ethanol are bound to pepsinogen transformation to the B form can take place. However, only four molecules of 1-butanol have to be bound to pepsinogen in order to facilitate this transformation. This is expected since the hydrophobic interaction with the longer hydrocarbon chain of 1-butanol will induce a greater conformational change on the pepsinogen molecule.

The constant K' can be regarded as a "transformation constant" since it increases with increasing the binding constant of the alcohol to pepsinogen, K_2, and with the rate of dissociation of C to B, k_3. The values of K' obtained from Figure 12 markedly increase with chain length of the alcohol, which indicate again the strong dependence of the inactivation potential of the alcohol on its chain length.

As the form B of pepsinogen is stable in acid, a transition from A to B in acid is, in principle, possible. However, this transformation competes with the very rapid process of activation by which A is transformed to pepsin, and is therefore of low probability. One may speculate that the A form of pepsinogen in acid is of a very unstable conformation with a high potential strain energy which provides the energy of activation required for the transformation to pepsin.

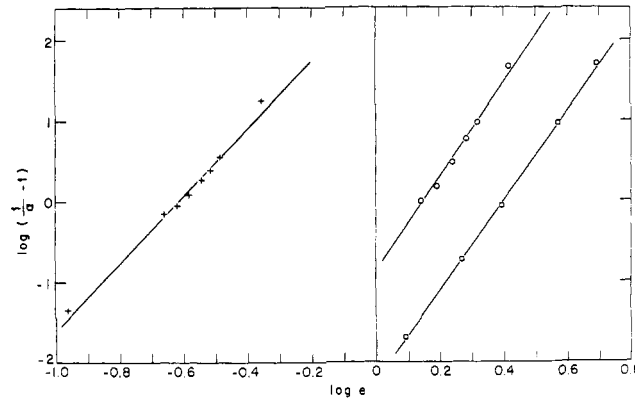

FIGURE 12: Plots of $\log[1/\alpha - 1]$ *vs.* $\log e$ for methanol (□—□) from data given in Figure 1; for ethanol (○—○) from data given in Figure 2; and for 1-butanol (×—×) from data given in Figure 3. α is the apparent fraction of pepsin activity and e is the total concentration of the alcohol in the inactivated pepsinogen solution.

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References

Anson, M. L. (1948), Crystalline Enzymes, New York, N. Y., Columbia University Press, p 305.

Arnon, R., and Perlmann, G. E. (1963), *J. Biol. Chem. 238*, 653.

Cohn, E. J. (1927), *J. Amer. Chem. Soc. 49*, 173.

Davidson, B., and Fasman, G. D. (1967), *Biochemistry 6*, 1616.

Fasman, G. D. (1962), *Methods Enzymol. 6*, 928.

Foss, T. G., and Schellman, J. A. (1964), *J. Chem. Eng. Data 9*, 551.

Grizzuti, K., and Perlmann, G. E. (1969), *J. Biol. Chem. 244*, 1764.

Herriott, R. M. (1938), *J. Gen. Physiol. 21*, 501.

Herskovits, T. T., Gadegbeku, B., and Jaillet, H. (1970), *J. Biol. Chem. 245*, 2588.

International Critical Tables (1930), Vol. 7, New York, N. Y., McGraw-Hill, pp 13, 67.

Ouchterlony, O. (1948), *Acta Path. Microbiol. Scan. 25*, 185.

Perlmann, G. E. (1963), *J. Mol. Biol. 6*, 452.

Perlmann, G. E. (1964), *Biopolym. Symp. 1*, 383.

Perlmann, G. E. (1967), *Advan. Chem. Ser. 63*, 268.

Perlmann, G. E. (1970), *in* Structure and Function Relationship of Proteolytic Enzymes, Neurath, H., and Ottesen, M., Ed., Copenhagen, Munksgaard, p 261.

Rajagopolan, T. G., Moore, S., and Stein, W. H. (1966), *J.*

Biol. Chem. 241, 4940.
Ryle, A. P. (1970), *Methods Enzymol. 19*, 316.
Ryle, A. P., and Porter, R. R. (1959), *Biochem. J. 73*, 75.
Tang, J. (1965), *J. Biol. Chem. 240*, 3810.
Tanksley, T. D., Neumann, H., Lyman, C. M., Pace, C. N., and Prescott, J. M. (1970), *J. Biol. Chem. 245*, 6456.

A Homologous Series of Affinity Labeling Reagents and Their Use in the Study of Antibody Binding Sites*

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ABSTRACT: A series of new radioactive affinity-labeling reagents for anti-DNP antibodies was synthesized. The chemically reactive group in these reagents (bromoacetyl derivatives of DNP haptens) is situated at an increasing distance from the DNP ring. This series thus provides a useful tool for the systematic mapping of antibody binding sites. Each of the reagents reacts specifically with the combining site of anti-DNP antibodies as shown by a variety of chemical and physicochemical criteria, such as stoichiometric bonding to a specific antibody only, and protection of the site by DNP haptens. Using a homologous series of reagents it was possible to show that both tyrosine and lysine become labeled and are apparently present at the combining sites of goat anti-DNP antibodies. The distribution of the label between these two amino acids was different with antibodies from different animals and is probably an individual trait of tne animal.

Affinity labeling of antibodies, initiated by Singer and his colleagues (Wofsy *et al.*, 1962; Metzger *et al.*, 1963), has become an important tool in studying the structural features of the antibody combining site. Studies with diazonium salts of various benzenoid haptens showed that in most cases tyrosines (in both heavy and light chains) are the residues labeled (Singer *et al.*, 1967; Good *et al.*, 1968). An indication that lysine residues might also be present at the combining site of antibodies was obtained by affinity labeling of anti-DNP antibodies with FDNB[1] (Shaltiel and Givol, 1967; Givol *et al.*, 1969). This was strongly supported by the finding that the mouse myeloma protein 315 which has anti-DNP activity could be labeled either at a tyrosine residue in its light chain or at a lysine residue in its heavy chain (Haimovich *et al.*, 1970).

In most of the affinity-labeling reagents, the chemically reactive group can form a covalent bond with only a few of the amino acid side chains that might be present at the binding site. One of the ways to overcome this difficulty was introduced by Fleet *et al.* (1969). These investigators made use of photochemically reactive affinity-labeling reagents which can tag any of the amino acid side chains. In the present study we wish to report another approach, namely the use of an homologous series of reagents in which the chemically reactive group is situated at increasing distances from the haptenic group. Such a series provides a useful tool for locating functional groups at various positions in and around the binding site. This paper describes the synthesis of such an homologous series of reagents, demonstrates the specificity of their reaction, and illustrates the potential uses of such a series for studying the differences in the combining sites of antibodies of the same specificity produced in different individual animals.

Materials and Methods

Synthesis of Reagents. *N*-DNP,*N'*-Z-ETHYLENEDIAMINE. *N*-Z-Ethylenediamine hydrochloride was prepared according to the method of Lawson *et al.* (1968). A solution of 2.3 g (10 mmoles) of *N*-Z-ethylenediamine hydrochloride in 15 ml of water was treated with 2.72 g of FDNB (15 mmoles) in 15 ml of ethanol in the presence of an excess of $NaHCO_3$. The reaction mixture was stirred for 2 hr at room temperature, and the precipitate formed was filtered and washed with water, then with 80% ethanol, and finally with ether. The compound was recrystallized from ethyl acetate: yield, 3 g (83%); mp 133°. *Anal.* Calcd for $C_{16}H_{16}N_4O_6$: C, 53.33; H, 4.44; N, 15.55. Found: C, 53.50; H, 4.53; N, 15.41.

N-DNP-ETHYLENEDIAMINE HYDROBROMIDE. A solution of 1.8 g (5 mmoles) of *N*-DNP-*N'*-Z-ethylenediamine in 10 ml of glacial acetic acid was mixed with 15 ml of HBr in CH_3COOH (45%). The reaction was allowed to proceed for 15 min, then stopped by addition of dry ether. The precipitate was washed with dry ether: yield, 1.4 g (90%); mp over 250°. *Anal.* Calcd for $C_8H_{11}BrN_4O_4$: C, 31.28; H, 3.61; N, 18.24. Found: C, 31.50; H, 3.40; N, 17.97.

N-BROMOACETYL-*N'*-DNP-ETHYLENEDIAMINE (BADE) (See Chart I). *N*-DNP-ethylenediamine hydrobromide (0.77 g, 2.5 mmoles) was suspended in 10 ml of dioxane containing 2.5 ml of 1 N NaOH and 5 ml of 1 N $NaHCO_3$ was added. This mixture was reacted with 0.6 g (2.5 mmoles) of *N*-hydroxysuccinimide ester of bromoacetic acid (Cuatrecasas *et al.*, 1969) which was dissolved in dioxane. The reaction was

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[1] Abbreviations used are: FDNB, fluorodinitrobenzene; BADE, *N*-bromoacetyl-*N'*-DNP-ethylenediamine; BADL, N^{α}-bromoacetyl-N^{ϵ}-DNP-lysine; BADB, N^{α}-bromoacetyl-N^{γ}-DNP-diamino-L-butyric acid; BADO, N^{α}-bromoacetyl-N^{δ}-DNP-L-ornithine; IADL, N^{α}-iodoacetyl-N^{ϵ}-DNP-L-lysine; Z, benzyloxycarbonyl.