2. EXPERIMENTAL
2.1 MATERIALS

Special reagents obtained from commercial sources were: Porcine Pepsin 2 x crystallized 2500-3200 units per mg protein, Pepstatin, Bovine Hemoglobin (Type II), Human Immunoglobulin G, N-Acetyl Phenylalanyl-3,5-diido-L-tyrosine and Glutaraldehyde 25% aqueous solution (grade II) (Sigma Chemicals Company, USA); Sephadex G-200 (Pharmacia Fine Chemicals Inc., Sweden); Casein and Bactoagar (Difco Laboratories, USA); Polyethyleneimine acetonitrile and Sodium borohydride (BDH, England); Cyanogen bromide (Sisco Research Laboratory, India). All the other chemicals used were of analytical grade and were obtained from BDH (India), E. Merck (India), Glaxo Laboratories (India) and Baker (U.S.A.).
2.2 METHODS

2.2.1 ENZYME IMMOBILIZATION

Pepsin was immobilized by chemical aggregation using glutaraldehyde as crosslinking agent, by covalent attachment to cyanogen bromide activated Sephadex G-200 and by adsorption to polyethyleneimine coated cotton cloth followed by crosslinking with glutaraldehyde.

2.2.1.1 Preparation of Insolubilized Pepsin Using Glutaraldehyde as Cross Linking Agent

The insolubilized pepsin was prepared by the method of Habeeb (1967) with certain modifications. One gram pepsin was dissolved in 20 ml of 0.1% sodium acetate buffer, pH 4. 6.25 g ammonium sulfate for 50% (w/v) saturation was added to precipitate the protein and affect conjugation of the amorphous particles. The solution was placed in a refrigerator at 4°C for four hours and 1.6 ml of 25% aqueous glutaraldehyde solution was added to make the final concentration of glutaraldehyde 2% (v/v). In order to facilitate crosslinking, the solution was placed in a mechanical shaker at room temperature for two hours. To remove the excess glutaraldehyde, the solution was allowed to remain in contact with 1.2 g sodium bisulfite for one hour. The solution was centrifuged at 2000 rpm at room temperature and the precipitate was washed with
saline (0.85% NaCl) until free of yellow colour. To the precipitate, 20 ml of 0.1M sodium acetate buffer, pH 4.0, was added and this suspension was treated with 25 mM sodium borohydride for three minutes to saturate the cross-linked product (Figure 3). After three minutes, the precipitate was repeatedly washed with 0.1M sodium acetate buffer, pH 4, and homogenized in a mortar Elvehjem homogenizer with a loosely fitting Teflon for sixty seconds. The insolubilized preparation was finally washed with 0.1M sodium acetate buffer, pH 4, until free of sodium chloride (AgNO₃ test) and stored at 4°C suspended in the same buffer and used as insoluble pepsin. The amount of protein cross-linked with glutaraldehyde was determined as described under Section 2.2.4.3.

2.2.1.2. Immobilization of Pepsin on Cyanogen Bromide Activated Sephadex G-200.

Sephadex G-200 was activated as described by Porath et al. (1967). 5 gm Sephadex was washed thoroughly with distilled water in a sintered glass funnel. The gel was sucked dry and suspended in 150 ml of 0.1M carbonate-bicarbonate buffer, pH 10, and mixed thoroughly by placing on a magnetic stirrer. 500 mg cyanogen bromide dissolved in 1.0 ml acetonitrile was added to the beaker containing Sephadex and mixed well in cold. The pH was maintained at 10 with sodium hydroxide. After 6 minutes, when the pH got stabilized at 10, the whole mass was transferred immediately to
a glass sintered funnel and washed with 0.1M carbonate-bicarbonate buffer, pH 10. After thorough washing, the activated Sephadex was suspended in 150 ml of pepsin solution (5 mg/ml acetate buffer, pH 4, 0.1M). The suspension was stirred on a magnetic stirrer at 4°C for 24 hours. In order to render the remaining activated polymer inactive, 0.1M glycine (1.126 gm in 150 ml) was added. After treatment with glycine for one hour, the enzyme-polymer was washed thoroughly with 0.1M acetate buffer, pH 4, and then suspended in 150 ml of the same buffer. The supernatant and washings were pooled to determine the amount of bound protein as described under Section 2.2.4.3.

2.2.1.3 Binding of Pepsin to Polyethyleneimine coated Cotton Cloth

0.3 gm of 50% polyethyleneimine was dissolved in 150 ml distilled water to make its concentration to 0.1% and its pH was adjusted to 7.0 with 0.1N HCl. To this 0.1% solution of polyethyleneimine, were added 40 cotton cloth square pieces 3 inches x 3 inches in size, and were left to soak it for 30 minutes at room temperature. This was done to activate the cotton cloth with polyethyleneimine. The cloth pieces were then washed with distilled water and air dried. 200 ml of pepsin solution (5 mg/ml) in 0.1M acetate buffer, pH 4, was taken in a beaker and the activated cotton cloth squares were
put in it. The beaker was then kept overnight at $4^\circ$C with constant slow shaking. Subsequently, 16 ml of 25% (w/v) glutaraldehyde was added to make the final concentration of glutaraldehyde to 2% (w/v). The solution was kept at room temperature for two hours with constant shaking. This helped in crosslinking the adsorbed pepsin. The enzyme bound cloth was then washed with 0.1M acetate buffer, pH 4, after which it was blotted dry and stored at $4^\circ$C for use. The washings were collected to determine the amount of protein adsorbed to the cloth as described under Section 2.2.4.3.

2.2.2 ENZYME ASSAYS

The following standard procedures were used for the determination of soluble as well as insoluble pepsin activities. While assaying the insoluble preparations, the assay mixtures were continuously agitated mechanically.

2.2.2.1 Assay of Peptic Activity Using Hemoglobin as Substrate.

With some modifications, pepsin was assayed essentially by the procedure described by Anson (1939). 0.3 ml of 0.1M KCl-HCl buffer (pH 2) was preincubated at $37^\circ$C for 5 minutes with 0.5 ml of 2% acid denatured Hb at pH 2 dissolved in the same buffer. This was then reincubated at $37^\circ$C with
either 0.2 ml of suitably diluted native pepsin or the same amount of insoluble pepsin in 0.1% KCl-HCl buffer pH 2, in a centrifuge tube, for 15 minutes. The reaction was stopped by the addition of 0.5 ml of 20% (w/v) trichloroacetic acid and the undigested Hb was removed by centrifugation at 3000 rpm for 10 minutes. In the case of pepsin bound to cotton cloth, 2.5 ml of 0.1% KCl-HCl buffer, pH 2, and 5 ml of acid denatured 2% Hb, pH 2, was added to one square of enzyme-bound cloth and incubation was done for 15 minutes at 37°C. The reaction in this case was stopped by the addition of 2.5 ml of 20% TCA. Control tubes were also run, in which TCA was added to the assay mixture before the addition of hemoglobin.

The extent of hydrolysis was determined in a suitably diluted aliquot by the procedure of Lowry et al. (1951), using tyrosine as standard. Absorption of tyrosine of the TCA soluble oligopeptides was also measured directly at 280 nm in a Bausch and Lomb Spectronic 21 Model UV-D spectrophotometer.

One enzyme unit is defined as that amount of enzyme which releases 1 μmole of tyrosine per 15 minutes at 37°C. The specific activity is defined as enzyme units per milligram of protein.

2.2.2.2 Assay of Peptic Activity Using N-Acetyl Phenylalanyl-3-5-dilodo L-Tyrosine (APDT) as Substrate

For the assay of pepsin using APDT as the substrate, the method of Tang (1970) was modified. A 0.002N substrate
sites, namely, intraperitoneal, intramuscular and subcutaneous. Four injections were given at each site. After a rest period of 10 days, a second injection containing 9 mg protein in 1.8 ml emulsion was given. This time three injections were given at each site. After the fourth week, a third booster injection was given intravenously into the ear vein, containing 5 mg protein in 1.0 ml suspensions. Thus a total of 20 mg protein was given to the rabbit. Blood was taken out in the fifth week by cardiac puncture. The titer value of the antisera was determined as described in Section 2.2.5.2.

2.2.5.2 Quantitative Precipitin Titration

The antigen-antibody reactions involving $F(ab')_2$ fragments and antisera raised against these fragments were studied using quantitative precipitin titration following a published procedure (Heidelberger and Kendall, 1935).

To one ml of antiserum was added increasing concentrations of $F(ab')_2$ protein and the mixture was incubated for two hours at $37^\circ$C and then for two days at $4^\circ$C. The precipitate was collected by centrifugation and washed twice with 0.15 M cold saline and dissolved in 0.05M KCl-HCl buffer, pH 2. The total protein was determined as described in Section 2.2.4.2. The titer value (antibody concentration) was obtained by subtracting the Lowry positive value of the added antigen from the total protein of antigen-antibody complex.
2.2.5.3 **Immunodiffusion**

Experiments on double immuno diffusion were performed by Ouchterlony's method (1949). A 1.5% agar solution in saline containing 0.02% sodium azide was poured on a petridish. The central and peripheral wells were then made. The antiserum obtained from the rabbit as described in Section 2.2.5.1 was placed in the central well. The commercial \( F(ab')_2 \) against which the antiserum was raised, was placed in two of the peripheral wells, while in the other two peripheral wells was placed the \( F(ab')_2 \) obtained by cleavage of human IgG by the immobilized pepsin. The commercial \( F(ab')_2 \) (antigen) taken corresponded to its concentration at the equivalence zone. The petridish was kept at 37°C for two hours and then for three days at 4°C in a humid atmosphere.