3. RESULTS
Immobilization of pepsin by chemical aggregation using glutaraldehyde, by covalent attachment to CNBr-activated Sephadex G-200 and by adsorption followed by cross-linking to polyethyleneimine coated cotton cloth resulted in the formation of highly stable enzyme conjugates with considerable enzymic activity. The balance sheet of the three procedures of immobilization used for pepsin is given in Table II.

3.1 Pepsin Crosslinked with Glutaraldehyde

As described in Section 2.2.1.1, the treatment of the 50% (w/v) ammonium sulfate precipitated enzyme with glutaraldehyde resulted in the formation of a brownish yellow crosslinked insolubilized product with significant proteolytic activity. The activity shown by the insolubilized pepsin using acid-denatured hemoglobin as the substrate was 67% of the activity shown by the soluble pepsin. When a synthetic substrate, N-acetyl phenylalanyl-3,5-diodo L-tyrosine (APDT) was used, the activity displayed by the insolubilized pepsin preparation was 79% that of the soluble native enzyme. The yield of the crosslinked pepsin was 86% and the percent immobilization amounted to 96% (Table II). When the activity of the enzyme was measured as a function of substrate (acid denatured hemoglobin) concentration and of inhibitor (pepstatin) concentration, it
TABLE - II

Immobilization of Pepsin by Different Methods.

Pepsin was immobilized by crosslinking with glutaraldehyde, by covalent attachment to Sephadex and by adsorption followed by crosslinking on cotton cloth as described in Section 2.2.1. Washings in all the three cases were pooled and assayed for peptic activity as described in Section 2.2.2. Matrix bound enzyme activity was determined by directly measuring the activity of pepsin of a known amount of matrix.

<table>
<thead>
<tr>
<th>Method</th>
<th>Enzyme added per gm matrix (units) (A)</th>
<th>Enzyme in washings per gm matrix (B)</th>
<th>Bound enzyme per gm matrix (C) (A-B) (units)</th>
<th>Percent immobilization (D) (C/A x 100)</th>
<th>Activity on matrix per gm (units) (E)</th>
<th>% Yield (E/C x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crosslinking with glutaraldehyde</td>
<td>9075</td>
<td>380</td>
<td>8695</td>
<td>96</td>
<td>7502</td>
<td>86</td>
</tr>
<tr>
<td>2. Covalent linking to Sephadex</td>
<td>39600</td>
<td>26400</td>
<td>13200</td>
<td>33.33</td>
<td>4555</td>
<td>34.5</td>
</tr>
<tr>
<td>3. Enzyme-cloth complex</td>
<td>61875</td>
<td>38610</td>
<td>23265</td>
<td>37.6</td>
<td>2920</td>
<td>12.55</td>
</tr>
</tbody>
</table>
was seen that the $K_m$ and $K_i$ values of the insolubilized pepsin were enhanced in comparison to those of free pepsin (Table III). The insolubilized pepsin had good storage stability too. It was found to possess 58% of the original proteolytic activity after 160 days of storage at 4°C (Table VI).

3.2 Pepsin Linked Covalently to CNBr-activated Sephadex G-200

Pepsin was coupled covalently to the activated Sephadex through its N-terminal amino group and the ε-amino group of its single lysine residue (Figure 2) as described in Section 2.2.1.2. This resulted in a white gelatinous mass with considerable peptic activity. Compared to the native pepsin, the activity of the immobilized enzyme was 52%. The yield of the immobilized enzyme was 34% and the percent immobilization amounted to 33% (Table II). In this case also, the $K_m$ and $K_i$ values were found to be enhanced as compared to the native pepsin (Table III). A slight enhancement was also seen in the value of energy of activation, as compared to that of native pepsin (Table III). The reusability and storage stability of this immobilized preparation were excellent. It lost almost none of its activity when stored at 4°C for more than five months (Table VI).
### TABLE - III

**Kinetic Parameters of Immobilized Pepsin Preparations.**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$K_m$ (x$10^{-5}$M)</th>
<th>$V_{max}$ (x$10^{-11}$M)</th>
<th>$K_i$ (x$10^{-11}$M)</th>
<th>$E_{act}$ (cal per mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crosslinked pepsin</td>
<td>5.3</td>
<td>.2187</td>
<td>7.29</td>
<td>80198</td>
</tr>
<tr>
<td>CNBr-Sephadex bound pepsin</td>
<td>4.28</td>
<td>.4516</td>
<td>5.50</td>
<td>66785</td>
</tr>
<tr>
<td>Cloth bound pepsin</td>
<td>3.0</td>
<td>.6666</td>
<td>4.95</td>
<td>53730</td>
</tr>
<tr>
<td>Native pepsin</td>
<td>3.07</td>
<td>.2828</td>
<td>4.45</td>
<td>61358</td>
</tr>
</tbody>
</table>
### TABLE - IV

**Reusability of Immobilized Pepsin.**

Appropriate amounts of immobilized enzyme were used for this study. At the end of each assay, the enzyme preparations were thoroughly washed with buffer. Fresh assay mixture was then added and peptic assay was performed as described in Section 2.2.2.

<table>
<thead>
<tr>
<th>Assay Number</th>
<th>Crosslinked pepsin</th>
<th>CNBr-Sephadex bound pepsin</th>
<th>Cloth bound pepsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>98</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td>6</td>
<td>98</td>
<td>95</td>
<td>96</td>
</tr>
<tr>
<td>7</td>
<td>98</td>
<td>92</td>
<td>95</td>
</tr>
</tbody>
</table>
3.3 **Pepsin Immobilized on Cotton Cloth**

Pepsin immobilized on polyethyleneimine coated cotton cloth was very convenient to handle and also very easy to store. The enzyme bound cloth showed 81% activity in comparison to native pepsin, when casein was used as the substrate. A lowering of \( K_m \) and energy of activation was seen, while the \( K_i \) of the preparation was increased (Table-III). The immobilized pepsin was used effectively in a column for the hydrolysis of casein. It could also be reused repeatedly for a number of assays (Table IV). The preparation was highly stable because when stored for 160 days at 4°C, it retained all of its activity (Table VI).

3.4 **PROPERTIES OF THE IMMobilIZED PREPARATIONS OF Pepsin**

To evaluate the effectiveness of pepsin immobilized by intermolecular crosslinking, on CNBr-activated Sephadex G-200 and on polyethyleneimine coated cotton cloth, several properties of the enzyme were investigated and compared with those of the native pepsin.

3.4.1 **Effect of pH**

The pH-activity profiles of the native and various immobilized preparations of pepsin are shown in Figure 6. When activity was measured as a function of pH, there was no
Figure 6. Effect of pH on the proteolytic activity of pepsin.

Hemoglobin solutions were prepared in buffers of the indicated pH. The activities of native and immobilized pepsin were determined using these buffer solutions. The buffers used for the various pH ranges were: KCl-HCl (pH 1.2), sodium citrate (pH 3), sodium acetate (pH 4.5), sodium phosphate (pH 6). The strength of all the buffer solutions was 0.1M.

(○-○) Native pepsin
(●-●) Crosslinked pepsin
(▲-▲) Covalently linked pepsin
(△-△) Adsorbed pepsin
change in the optimal pH in the case of crosslinked pepsin and pepsin immobilized on cotton cloth from that of the native pepsin. However, the optimum pH of the covalently linked pepsin to Sephadex shifted by 0.5 unit towards the acidic side.

There was also no significant broadening of the pH activity curve except in the case of pepsin bound to Sephadex G-200. The pH dependence of the activity of pepsin with hemoglobin as substrate is comparable with earlier reported values (Tomono et al. 1981; Northrop, 1922). All the three immobilized pepsin preparations showed more activity than the native pepsin at higher pH values.

3.4.2 pH Stability

Pepsin becomes rapidly and completely inactivated in the pH region above 6.0 (Bovey and Yanari, 1960). It was therefore considered of interest to investigate whether the insoluble preparations of the enzyme exhibited higher resistance to pH denaturation. Figure 7 shows the effect of incubation at pH 4 of native and immobilized pepsin preparations. The immobilized pepsin was highly resistant to this pH value and while the crosslinked pepsin lost 4% of its activity after 120 minutes incubation, the other two preparations retained all their activity. The native pepsin, how-
Figure 7. pH-stability of the immobilized enzyme.

Native and immobilized preparations of pepsin were incubated in 0.1M sodium-acetate buffer at pH 4. Aliquots were removed at indicated intervals and adjusted to the optimum pH before activity of the enzymes was determined.

( O-O ) Native pepsin
( •-• ) Crosslinked pepsin
( ▲-▲ ) Covalently-linked pepsin
( △-△ ) Adsorbed pepsin
ever, lost its activity rapidly in the first 90 minutes incubation and had only 30% of its activity left after 120 minutes. This indicated that the immobilized pepsin preparations had become highly resistant to pH changes.

3.4.3 Effect of Temperature on the Reaction Rate

The effect of temperature on the reaction rate is shown in Figure 8. As evident from the figure, immobilization of pepsin did not result in any shift of the optimum temperature at 60°C. The $Q_{10}$ values for soluble and all immobilized pepsin preparations are shown in Table V. The insolubilized enzyme retained more activity as compared to the native pepsin with increase in temperature. The Arrhenius plots of the same data (with the exclusion of the ones obtained at higher temperatures where the activity declined) yielded straight lines (Figure 9).

The activation energies calculated from the data plotted in Figure 8 are 80198, 66785, 53730 and 61358 calories per mole for crosslinked pepsin, CNBr-Sephadex G-200 bound pepsin, cloth bound pepsin and native pepsin, respectively (Table III). The equation

$$\frac{K_2}{K_1} = 0.219 E \frac{(T_2 - T_1)}{T_1 \times T_2}$$

was used to calculate these values, where $E$ is the energy
Figure 8. **Effect of temperature on the reaction rate**

Native and immobilized pepsin solutions were prepared in 0.1 M KCl-HCl buffer, and their activities were determined after assayed at the indicated temperatures.

- (○○) Native pepsin
- (●●) Crosslinked pepsin
- (▲▲) Covalently-linked pepsin
- (▲-▲) Adsorbed pepsin
### TABLE - V

**Q₁₀** Values of Native and Immobilized Preparations of Pepsin.

Native and immobilized preparations of pepsin were assayed at the indicated temperatures under standard assay conditions described in Section 2.2.2. Each value represents the average of at least two independent experiments.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Native pepsin</th>
<th>Crosslinked pepsin</th>
<th>Covalently linked pepsin</th>
<th>Adsorbed pepsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 - 30</td>
<td>1.69</td>
<td>3.23</td>
<td>1.67</td>
<td>1.32</td>
</tr>
<tr>
<td>30 - 40</td>
<td>1.61</td>
<td>1.64</td>
<td>1.86</td>
<td>1.29</td>
</tr>
<tr>
<td>40 - 50</td>
<td>1.13</td>
<td>1.34</td>
<td>1.29</td>
<td>1.11</td>
</tr>
<tr>
<td>50 - 60</td>
<td>1.12</td>
<td>1.16</td>
<td>1.19</td>
<td>1.11</td>
</tr>
<tr>
<td>60 - 70</td>
<td>0.21</td>
<td>0.35</td>
<td>0.17</td>
<td>0.78</td>
</tr>
</tbody>
</table>

The **Q₁₀** values represent the ratio of the velocity at temperature \((T + 10)°C\) to that at **T °C**.
Figure 9. **Arrhenius Plot**

The data from Figure 8 was used.

- (○-○) Native pepsin
- (●-●) Crosslinked pepsin
- (▲-▲) Covalently-linked pepsin
- (△-△) Adsorbed pepsin
of activation, $T_1$ and $T_2$ are the absolute temperatures and $K_1$ and $K_2$ are the activities of the enzyme at these temperatures. It was seen that although the activation energy of crosslinked pepsin and CNBr-treated Sephadex bound pepsin is greater than that of native pepsin, immobilization of pepsin on polyethyleneimine coated cotton cloth resulted in a decrease in the energy of activation.

3.4.4 Heat Stability

In order to investigate the stabilization conferred by immobilization, the thermal inactivation of native and the insoluble preparations of pepsin was determined at 65°C (Figure 10). The native pepsin lost its activity rapidly and continuously, till at the end of two hours incubation at 65°C, it had only 13% of its activity left. On the other hand, under similar experimental conditions, the three immobilized preparations of pepsin retained significant amounts of activity. This showed that pepsin after immobilization had become more stable at higher temperatures than the native one.

As shown in Figure 11, the insolubilization greatly enhanced the stability of the enzyme at elevated temperatures. On preincubation at 70°C for 15 minutes, before the assay at 37°C, the native pepsin had only 21% of its original acti-
Figure 10. **Thermal stability of the immobilized pepsin.**

Native and immobilized pepsin in 0.1M KCl-HCl buffer pH 2.0 were incubated at 65°C. Aliquots were removed at various intervals of time, chilled immediately, and activity was determined at 37°C.

(○-○) Native pepsin  
(○-○) Crosslinked pepsin  
(▲-▲) Covalently-linked pepsin  
(▲-▲) Adsorbed pepsin
Preincubation Time (min)

% Remaining Activity

Preincubation Time (min)
Figure 11. **Effect of temperature on the stability of the enzyme.**

Native and immobilized pepsin solutions were prepared in 0.1M KCl-HCl buffer, pH 2. These were incubated at the indicated temperatures for 15 minutes; activity was then determined at 37°C as described in Section 2.2.2.

(○○) Native pepsin
(■■) Crosslinked pepsin
(▲▲) Covalently-linked pepsin
(△△) Adsorbed pepsin
ivity left. The crosslinked pepsin, the covalently bound enzyme and pepsin bound to cloth retained 50%, 84% and 90% of their respective activities under similar experimental conditions.

3.4.5 Effect of Urea

The effect of urea concentrations on the activity of immobilized and native pepsin towards acid-denatured hemoglobin and APDT are shown in Figures 12 and 13 respectively. Using hemoglobin as the substrate, the native enzyme showed a slight increase in activity as the concentration of the denaturant was elevated. However, the crosslinked pepsin and the cloth bound pepsin were considerably activated under similar conditions, having 181% and 140% activity in 10M urea. The covalently immobilized pepsin on Sephadex G-200 showed no change in its activity.

When APDT was used as the substrate for enzyme assay (Figure 13), the native pepsin as well as all the immobilized preparations of the enzyme showed no variation in their activities. Even on incubation with 8M urea for 20 minutes, the enzyme preparations retained 100% activity.

3.4.6 Stability Against Urea

The effect of incubation of native and the immobilized preparations of pepsin with 4M urea for varying intervals
Figure 12. **Effect of urea on the proteolytic activity.**

Different solutions of native and immobilized pepsin were prepared in 0.1M KCl-HCl buffer (pH 2) and urea was added to make their concentration as indicated in the figure. These solutions were incubated at 37°C for 20 minutes and then their activities were determined using acid-denatured hemoglobin as substrate.

- (o-o) Native pepsin
- (e-e) Crosslinked pepsin
- (▲▲) Covalently-linked pepsin
- (▲-▲) Adsorbed pepsin
Figure 13. Effect of urea on the activity of native and immobilized enzyme.

Different solutions of native and immobilized pepsin were prepared in 0.1M KCl-HCl buffer, pH 2, and urea was added to make their concentration as indicated in the figure. These solutions were incubated at 37°C for 20 minutes and their activities were determined using APDT as substrate.

- (○-○) Native pepsin
- (●-●) Crosslinked pepsin
- (▲-▲) Covalently-linked pepsin
- (△-△) Adsorbed pepsin
of time was determined. As is evident from Figure 14, the native pepsin and the pepsin immobilized on Sephadex showed no effect on the activity in 4M urea upon incubation for two hours. The cloth bound pepsin showed a 10% increase in activity. In the case of crosslinked pepsin, the activity increased to 142% in the first 60 minutes incubation and remained constant thereafter. From these data, it is clear that immobilization has conferred substantial stability upon pepsin.

3.4.7 Effect of Guanidine-Hydrochloride on Enzyme Activity

The effect of Gdn-HCl concentration on the activity of immobilized and native pepsin towards acid-denatured hemoglobin is showed in Figure 15. At low Gdn-HCl concentration, the native pepsin lost a little activity. However, when the Gdn-HCl concentration was increased to 3M, the activity of native pepsin sharply declined and further increase in the concentration of Gdn-HCl resulted in complete inactivation of the enzyme. These results are comparable with earlier reports of Blumenfeld et al. (1970). Low Gdn-HCl concentration had an activating effect on the crosslinked and cloth bound pepsin. The activity decreased with increasing concentration of the denaturant, but none of the immobilized preparations was completely inactivated even by
Figure 14. Effect of preincubation time on the stability of immobilized and native pepsin in 4M urea.

Native and immobilized pepsin in 0.1M KCl-HCl buffer pH 2, were made 4M with respect to urea concentration. Aliquots were withdrawn at indicated intervals and activity was determined using acid-denatured hemoglobin as substrate.

( o-o ) Native pepsin
( e-e ) Crosslinked pepsin
( ▲▲ ) Covalently-linked pepsin
( △△ ) Adsorbed pepsin
Figure 15. Effect of guanidine-hydrochloride on the activity of pepsin.

Native and immobilized preparations of pepsin in 0.1M KCl-HCl buffer (pH 2) were taken and increasing amounts of Gdn-HCl was added to them. These solutions were incubated at 37°C for 20 minutes and then their activities were determined.

(○-○) Native pepsin
(●-●) Crosslinked pepsin
(▲-▲) Covalently-linked pepsin
(△-△) Adsorbed pepsin
10M Gdn-HCl. In 10M Gdn-HCl, the Sephadex-bound pepsin, the crosslinked pepsin and the cloth-bound pepsin retained 10%, 20% and 26% activity, respectively.

3.4.8 Stability Against Guanidine Hydrochloride

To test the stability of the immobilized pepsin preparation against guanidine hydrochloride, the native and immobilized pepsin was incubated with 3M Gdn-HCl for various time intervals, before the assay at 37°C. The results are shown in Figure 16. The native pepsin rapidly lost 96% activity after the first thirty minutes incubation, and further prolongation of the incubation time with the denaturant resulted in its complete inactivation. Under similar experimental conditions, the cross-linked and covalently-bound pepsin retained 30% and 10% of their respective activities after two hours of incubation. The cloth-bound pepsin, on the other hand, showed a 12% enhancement in its activity upon incubation with 3M Gdn-HCl.

3.4.9 Effect of Substrate Concentration

There was a continuous rise in enzyme activity with increasing substrate (acid-denatured hemoglobin) concentration for both native and the immobilized pepsin preparations (Figure 17). A lowering of $V_{max}$ was observed upon cross-
Figure 16. **Effect of preincubation time on the stability of native and immobilized enzyme in 3M guanidine-hydrochloride.**

Native and immobilized pepsin solutions were incubated in 0.1M KCl-HCl buffer, pH 2, and made 3M with respect to Gdn-HCl concentration. At the indicated intervals, 0.2 ml suspensions were taken out and activity was assayed.

- (○-○) Native pepsin
- (■-■) Crosslinked pepsin
- (▲-▲) Covalently-linked pepsin
- (△-△) Adsorbed pepsin
Figure 17. Effect of substrate concentration on the activity of native and immobilized enzymes.

Increasing hemoglobin concentrations from $3 	imes 10^{-5}$ M to $30 	imes 10^{-5}$ M were taken. Enzyme activity was determined at pH 2 with the substrate concentrations shown in the figure. The assay was done at 37°C.

( o-o ) Native pepsin
( e-e ) Crosslinked pepsin
( ▲▲ ) Covalently-linked pepsin
( ▲-o ) Adsorbed pepsin
linking of pepsin, while in the case of cloth bound pepsin and pepsin bound to Sephadex it was increased (Table III). The $K_m$ values as determined from the double reciprocal plot are also given in Table III.

3.4.10 Effect of Pepstatin

Pepsin, native and immobilized, was subjected to increasing concentrations of its inhibitor, pepstatin, before assaying for enzymic activity. All the three immobilized preparations were considerably more stable as compared to the native pepsin (Figure 18). The $K_i$ values of the native and immobilized pepsin preparations are given in Table III. The enhancement of $K_i$ upon immobilization indicated stabilization of the enzyme.

3.4.11 Reusability

The reusability of the immobilized preparations of pepsin was investigated for finding out its potential in preparative purposes. At the end of each assay, the enzyme was removed from the assay mixtures by centrifugation and washed with buffer before being mixed with the new assay mixture. This procedure was repeated without any appreciable loss of activity (Figure 19).
Figure 18. **Effect of Pepstatin**

Increasing concentrations of pepstatin (pepsin inhibitor) were used for the experiment, as shown in the figure. The native and immobilized pepsin preparations were incubated with the shown concentrations of pepstatin for 10 minutes. Acid denatured hemoglobin was added to start the reaction at 37°C.

- Native pepsin
- Crosslinked pepsin
- Covalently-linked pepsin
- Adsorbed pepsin
% Remaining Activity vs. Pepstation Concentration ($10^{-10}$ Moles)
Figure 19. **Reusability of immobilized pepsin.**

After each assay, the immobilized pepsin was washed and activity was measured using fresh assay mixture, with hemoglobin as substrate.

- (●-●) Crosslinked pepsin
- (▲-▲) Covalently-linked pepsin
- (▲-▲) Adsorbed pepsin
3.4.12 Storage Stability

Native and immobilized pepsin preparations were stored at 4°C and their activity determined at various time intervals. It was observed that after 160 days storage, the native pepsin had only 21% of its activity left. The crosslinked pepsin retained 58% activity during the same time period, while pepsin immobilized on CNBr-activated Sephadex and cotton cloth lost no activity (Table VI).

3.5 Laboratory Scale Columns

In order to study the efficiency of the immobilized pepsin preparations in transforming their substrates into products during continuous operations, laboratory scale columns of covalently linked pepsin and cloth bound pepsin were prepared and were used for continuous hydrolysis of casein and limited cleavage of human immunoglobulin G.

3.5.1 Hydrolysis of Casein

A column containing 22.4 mg bound pepsin to Sephadex G-200 was used. A 0.5% casein solution prepared in 0.1M acetate buffer, pH 4, was continuously passed through the column at a flow rate of 10 ml per hour. At 37°C, 50% hydrolysis of casein was observed (Figure 20). The column
TABLE - VI

Effect of Storage at 4°C on the Activity of Native and Immobilized Pepsin.

Native and immobilized pepsin preparations were stored at 4°C for the indicated periods before their activity was determined as described in Section 2.2.2.

<table>
<thead>
<tr>
<th>Storage time (days)</th>
<th>Native pepsin</th>
<th>CNBr-Sephadex bound pepsin</th>
<th>Cross-linked pepsin</th>
<th>Enzyme cloth complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>1</td>
<td>100</td>
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<td>5</td>
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<td>100</td>
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<tr>
<td>10</td>
<td>94</td>
<td>104</td>
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<td>100</td>
</tr>
<tr>
<td>20</td>
<td>85</td>
<td>98</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>78</td>
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<tr>
<td>60</td>
<td>68</td>
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<td>99</td>
</tr>
<tr>
<td>120</td>
<td>46</td>
<td>99</td>
<td>86</td>
<td>100</td>
</tr>
<tr>
<td>160</td>
<td>21</td>
<td>98</td>
<td>58</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 20. **Hydrolysis of Casein**

A column was packed with pepsin bound on cloth, containing 20 mg of the enzyme. Another one was packed with 22.4 mg of pepsin bound to Sephadex. They were equilibrated with 0.1M acetate buffer pH 4. Hydrolysis of casein was carried out for 21 days at 37°C.

(▲▼) Covalently-linked pepsin

(▲▼) Adsorbed pepsin
was operated for 21 days without any loss in enzyme activity.

Another column, packed with cloth-bound pepsin containing 20 mg of the enzyme, was also used for continuous hydrolysis of casein. The casein solution (0.5%) in 0.1M acetate buffer pH 4, that was passed through the column at a rate of 7 ml per hour at 37°C was 81% hydrolysed (Figure 20). Hydrolysis of casein through this column was also carried out for 21 days without any change in the activity of the enzyme in the column.

3.5.2 Limited Cleavage of Human IgG by Pepsin Immobilized on Sephadex

A column packed with pepsin immobilized on Sephadex G-200, containing 22.4 mg of the enzyme was used at 37°C. A 20 mg/ml solution of IgG in 0.1M acetate buffer/0.15N NaCl, pH 4, was passed through it at the rate of 7 ml per hour. The peptic digest, on analysis showed a very small initial rise in optical density, a major peak containing of F(ab')₂ fragments and another small peak consisting of pFc fragments. The elution profile of digest of IgG, cleaved by pepsin, is shown in Figure 21.

3.6 IMMUNOLOGICAL STUDIES

Results of the quantitative precipitation titration of rabbit anti F(ab')₂ antisera with the commercial F(ab')₂
Figure 21. **Cleavage of Immunoglobulin G.**

Digestion of IgG by pepsin immobilized on Sephadex G-200 was done as described in Section 2.2.3.2. The optical density of the digest fragments was measured directly at 280 nm.
are graphically shown in Figure 22. The titer value of the antiserum as determined by the quantitative precipitin titration came out to be 40 μg per ml antiserum.

The immunodiffusion patterns of the commercial $F(\text{ab}')_2$ and the $F(\text{ab}''')_2$ obtained by peptic cleavage are shown in Figure 23. The rabbit antiserum raised against the commercial $F(\text{ab}')_2$ solution was used in these experiments. As can be seen from the figure, the precipitin arcs obtained with commercial $F(\text{ab}')_2$ are similar to those obtained with the $F(\text{ab}')_2$ got from cleavage of IgG. The result confirmed that the $F(\text{ab}')_2$ fragments obtained from limited cleavage of IgG by the immobilized pepsin were pure.
Figure 22. **Quantitative Precipitation Titration of rabbit antiserum raised against commercial F(ab')₂.**

Details are described in Section 2.2.5.2. The antigen-antibody complex precipitate was dissolved in 0.1M KCl-HCl buffer, pH 2, and the protein estimated by Lowry's method as described in Section 2.2.4.2.
Antigen Concentration (µg)

Total protein content in the immuno-precipitate (µg)