A COLORIMETRIC METHOD FOR THE DETERMINATION OF
THE PROTEOLYTIC ACTIVITY OF DUODENAL JUICE

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The coupling of diazotized aryl amines with proteins in alkaline solution yield chromophoric protein derivatives. Digestion of a solution of such azoproteins with proteolytic enzymes results in the formation of colored components soluble in trichloroacetic acid. The intensity of the color in the trichloroacetic acid filtrate of the digested substrate is a function of the proteolytic activity of the enzyme solution and serves as the basis for the method here described.

EXPERIMENTAL

Preparation of Sulfanilamide-Azocasein—Solution A, 50 gm. of re-precipitated, fat-free casein are dissolved with stirring in 1 liter of water containing 10 gm. of NaHCO$_3$. Solution B, 5.0 gm. of sulfanilamide are dissolved in 200 ml. of H$_2$O containing 6.0 ml. of 5.0 N NaOH. 2.20 gm. of NaNO$_2$ are added. To the stirred solution 18.0 ml. of 5 N HCl are added and stirring is continued for 2 minutes. 18.0 ml. of 5 N NaOH are then added; the solution is stirred and added at once, with stirring, to Solution A.

The azoprotein is precipitated by acidification to pH 4.5, washed with water and alcohol, and air-dried.

The azocasein is a red-orange compound with an absorption maximum at 440 m$\mu$ (Fig. 1).

Substrate Solution—A stock solution of the substrate containing 25 mg. of azocasein and 5 mg. of sodium bicarbonate per ml. is prepared by dissolving 2.50 gm. of azoprotein in 50 ml. of 1.0 per cent NaHCO$_3$ at 60° with stirring. The pH is adjusted to 8.3 and the solution diluted to 100 ml. with distilled H$_2$O. The stock solution is stored at 0°.

Duodenal Juice—The specimens are centrifuged at about 1500 R.P.M. for 10 minutes and the sample taken from the relatively homogeneous middle layer. 1 ml. of sample is diluted to 100 ml. with bicarbonate buffer (5 mg. per ml.), pH 8.3.

Procedure

Each determination is set up in duplicate. The flask containing the substrate solution and a rack with the proper number of tubes are placed in a
water bath set at 38°. After the substrate solution has reached bath temperature, 1 ml. is pipetted into each of the tubes. 1 ml. of the diluted duodenal juice is then added to the tubes containing the substrate. A substrate blank is prepared by substituting 1 ml. of bicarbonate buffer for the test sample. At the end of 30 minutes the digestion is stopped and undigested azoprotein precipitated from the solution by the addition of 8 ml. of 5 per cent trichloroacetic acid to each tube, including the substrate blank tubes. The contents of each tube are filtered through paper. To a 5 ml. aliquot of the filtrate are added 5 ml. of approximately 0.5 N NaOH and the color is read on an Evelyn photoelectric colorimeter with the No. 440 filter. No fading of the color has been observed for at least 2 hours.

![Absorption curve of sulfanilamide-azo-casein (0.25 mg. per ml.)](image)

Fig. 1. Absorption curve of sulfanilamide-azo-casein (0.25 mg. per ml.)

It has not been found necessary to run a blank on the enzyme solution; after the total dilution of 1:2000 and treatment with trichloroacetic acid, even the darkest samples have always shown 100 per cent light transmission.

**Calculations**

The enzymatic hydrolysis of a protein is a first order reaction, the velocity constant of which is expressed by the equation

\[ K = \frac{1}{t} \cdot 2.3 \log \frac{C_1}{C_2} \]

where \(C_1\) and \(C_2\) are initial and final protein concentrations respectively after \(t\) minutes of digestion.
Since the color-concentration relationship of sulfanilamide-azocasein and its digestion products obeys Beer's law, optical density values may be substituted for $C$ values. The initial concentration, $C_1$, is determined by adding 5 ml. of $0.5 N$ NaOH to a 5 ml. aliquot of a 1:200 dilution of the substrate and then reading the color of the final solution. The $C_2$ value is determined by subtracting the optical density of the trichloroacetic acid filtrate from the $C_1$ value. This is permissible, since a solution of azocasein which is completely digested (no trichloroacetic acid precipitate) has the same color intensity as an undigested sample.

### Table I

Sample Calculation of $K$ Values

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Substrate blank</th>
<th>Duodenal Juice 1</th>
<th>Duodenal Juice 2</th>
<th>Undigested substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>92</td>
<td>58</td>
<td>51</td>
<td>50*</td>
</tr>
<tr>
<td>2</td>
<td>92</td>
<td>57</td>
<td>72</td>
<td>50*</td>
</tr>
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<td>92</td>
<td>58</td>
<td>72</td>
<td>50*</td>
</tr>
<tr>
<td>4</td>
<td>92</td>
<td>57</td>
<td>72</td>
<td>50*</td>
</tr>
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<td>92</td>
<td>58</td>
<td>72</td>
<td>50*</td>
</tr>
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<td>6</td>
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<td>57</td>
<td>72</td>
<td>50*</td>
</tr>
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<td>7</td>
<td>92</td>
<td>58</td>
<td>72</td>
<td>50*</td>
</tr>
<tr>
<td>8</td>
<td>92</td>
<td>57</td>
<td>72</td>
<td>50*</td>
</tr>
</tbody>
</table>

$*\text{The undigested substrate must be diluted 20 times more than the filtrate samples to be readable in the colorimeter; therefore, the optical density must be multiplied by 20 to yield the }C_1\text{ value.}$

The velocity constant is calculated for the diluted solution and multiplied by the dilution factor to obtain the reaction constant for the undiluted juice. A sample of the calculations appears in Table I.

In these calculations the blank value has been subtracted from the optical densities of the filtrates only to show the magnitude of the blanks. In practice, the instrument is set to 100 per cent transmission with the blank. With a direct reading instrument, such as the Klett, the calculations are simpler still.

### Results

If the enzymatic digestion of the azocasein conforms to the equation for a monomolecular reaction, then the velocity constant rather than some arbitrary unit may be used as a measure of enzyme activity. From the kinetic equation it may be seen that $\log (C_1/C_2)$ is directly proportional to $t$. 

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That this relation is followed under the conditions of the determination is shown in Fig. 2. With two dilutions of duodenal juice, a rectilinear relationship was found between \( \log (C_1/C_2) \) and time, \( t \), in minutes.

The velocity constant of an enzyme reaction is a function of enzyme concentration. The data plotted in Fig. 3 reveal that under the conditions of the assay enzyme activity (\( \log (C_1/C_2) \)) is directly proportional to the concentration in the duodenal juice. In the 1:50 dilution sample of Fig. 3, 10 per cent digestion had occurred. In other experiments the direct proportionality has been shown to exist in from 0 to 25 per cent digestion of the substrate, thereby permitting determinations to be made over a large range of dilutions. The direct relationship is especially satisfactory in that it permits recalculation to original juice strength merely by multiplication by the dilution factor.
DISCUSSION

The method described has proved readily adaptable to the routine testing of clinical samples. It is simple, rapid, and sensitive and conforms to the theoretical monomolecular reaction equation. It has shown a high order of reproducibility; from the fourteen determinations run to yield the data in Fig. 2, in which digestion time and enzyme concentration were varied, an average velocity constant of 0.1198 was calculated, with an average deviation of ±0.0031 or ±2.5 per cent. A definite advantage of this method over many in which suspensions of insoluble substrates are employed is that the digestion is not influenced by particle size. Since the color of azocasein follows Beer's law, optical densities may be substituted for protein concentration, thus eliminating the necessity for the construction of nomographs. The color is stable and chromogenic reagents are not required for its development. Slight variations in substrate concentration of the stock solution are of minor significance, since in each determination the substrate concentration is determined and considered in the calculations.

The technique, in which a chromophoric protein substrate is employed, may be extended to the determination of other proteolytic enzymes. Sulfanilamide-azocasein is insoluble at acid pH and therefore unsuitable for peptic analysis. By the proper choice of aryl amine and protein, substrates may be prepared which are suitable for proteolytic enzymes in general. Further investigations are now in progress.

SUMMARY

A colorimetric method for the determination of tryptic activity has been presented in which a soluble chromophoric protein substrate, sulfanilamide-azocasein, is employed.

The increase in color in a trichloroacetic acid filtrate of the digestion mixture is a function of enzyme activity.

The enzymatic hydrolysis satisfies the conditions for a monomolecular reaction and permits activity to be expressed in terms of the velocity constant.
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