CHOLESTEROL ESTERASES

I. PREPARATION OF SUBSTRATE MIXTURE AND CHARACTERIZATION OF THE HYDROLYTIC CHOLESTEROL ESTERASE OF PANCREATIN*

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The enzyme or enzymes involved in the formation and hydrolysis of cholesterol esters have been the subject of sporadic studies since 1910 when Kondo (1) reported that extracts of horse and ox liver catalyzed the hydrolysis of these esters. The most extensive studies have been carried out by Sperry and coworkers (2) who also reviewed the earlier literature and pointed out the lack of agreement between several of the reports. A critical review of the previous work will show the following points which are of interest in connection with the studies on cholesterol esterase in progress in our laboratory. It has not been clearly shown whether the synthesis and hydrolysis of cholesterol esters are catalyzed by a single enzyme or by different enzymes. The enzyme activities related to cholesterol esters have not been definitely distinguished from the activity of other esterases occurring in the same tissues. The identity of the enzymes reported in various tissues such as liver, blood, and pancreas has not been established. There has not been an adequate characterization of the enzyme from any of the reported sources.

In a majority of the studies serum has been used to supply the substrate. This has been unfortunate for several reasons. This substrate mixture contains the end-products of the reaction irrespective of whether the synthesizing or hydrolyzing activity is under study. Also this substrate mixture has been reported to possess both kinds of activity, depending on the experimental conditions. Moreover, the use of serum limits the types of controls and experimental conditions which may be employed. In general the studies with serum have suggested a low order of activity for the enzyme; in many cases the incubation time has been extended to 48 or 72 hours in order to demonstrate appreciable synthesis or hydrolysis. The small amount of substrate, usually of the order of 1 mg. or less, has also introduced the possibility of considerable error in apparent activity due to small errors in the determination of cholesterol.

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Since the experiments described below were completed, Nieft and Deuel (3) and Nieft (4) have reported studies on the cholesterol esterases of rat liver and intestine. The existence of enzyme systems in both organs for synthesizing and hydrolyzing cholesterol esters has been verified. The esterifying system requires the presence of phosphate ion and a fatty acid source. The activity of the hydrolytic system was accentuated by the presence of soya lecithin. Also the hydrolytic system of rat intestine was found to consist of at least two factors.

In planning a detailed and extensive study of the synthesis and hydrolysis of cholesterol esters it seemed necessary first to devise substrate mixtures which could be employed in a variety of different types of experiments, and secondly, to characterize the catalytic activity from some easily obtainable standard source to provide a basis for assaying various tissues and fluids for activity and for comparing the activity from various sources. The present report presents our data on a suitable substrate mixture for studying the enzymatic hydrolysis of cholesterol esters and a characterization of the hydrolytic activity of a commercial pancreatin.

**EXPERIMENTAL**

*Preparation of Cholesterol Oleate*—The ester was prepared according to the directions of Page and Rudy (5). The product, which was obtained in 85 per cent yield, melted at 44.0° and gave no precipitate with digitonin. For ease in handling it was stored in a vacuum desiccator in the refrigerator, for at room temperature the crystals gradually became amorphous and sticky.

*Preparation of Substrate Mixture*—A weighed amount (100 mg. or less) of cholesterol oleate is placed in each of a series of test-tubes (25 X 200 mm.), previously tested for use with a stainless steel pestle of the Potter-Elvehjem homogenizing apparatus (6), and 0.5 cc. of ethyl ether is added to dissolve the ester. The following reagents are then added to each tube in order: 1 cc. of 10 per cent sodium glycocholate, 10 cc. of 0.154 M potassium phosphate buffer, 1 cc. of 1:1000 merthiolate, and 500 mg. of egg albumin, impalpable powder, soluble. The contents of the tubes are then homogenized for 1 minute. Following this the tubes are placed in a specially designed shaking apparatus at 37° for 1 hour. The tubes are moved through a vertical arc of approximately 55° at a rate of 100 excursions per minute while immersed in a constant temperature bath. After the preliminary shaking period which brings the temperature of the substrate mixture to that of the bath and removes the ether, the tubes are removed and 1 cc. of enzyme solution or substitute is added to each tube. The contents are mixed well by hand and two samples (1 cc. or less) are removed for determination of zero hour values. The tubes are immediately
returned to the shaker. The volume of solution in the tubes is not allowed to fall below 7 cc. during an experiment; so when it is necessary to remove more than 8 cc. of samples the amounts of all the constituents are doubled.

We routinely determined both the total and free cholesterol content of the samples by the method of Schoenheimer and Sperry (7) with the modification of Sperry (8). The determination of both fractions of cholesterol allowed for calculations of the per cent hydrolysis of the ester on the basis of the increase in free cholesterol or the decrease in cholesterol ester and in addition provided data (total cholesterol content) for evaluation of the uniformity of the emulsion during incubation. Substrate mixtures prepared as described above and shaken for the duration of an experiment were uniform in total cholesterol content up to 48 hours. In general the total cholesterol content of aliquots did not vary more than

<table>
<thead>
<tr>
<th>Digest No.</th>
<th>Total cholesterol during incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr.</td>
</tr>
<tr>
<td>1</td>
<td>16.0</td>
</tr>
<tr>
<td>2</td>
<td>15.7</td>
</tr>
<tr>
<td>3</td>
<td>49.7</td>
</tr>
<tr>
<td>4</td>
<td>50.4</td>
</tr>
</tbody>
</table>

duplicate analyses on the same aliquot. This result indicates that, within the limits of accuracy of the method used for determining the cholesterol, the mixtures are uniform in composition throughout the duration of an experiment. Occasionally, the total cholesterol content of a single sample differed markedly from the average of the others obtained from the same digest. These were very likely due to sampling errors and were rejected from the calculations. The total cholesterol content of samples removed after 0, 3, 12, and 24 hours of incubation of representative digests is given in Table I.

During the development of the substrate mixture a large number of emulsifying agents were tested singly and in various combinations. Sodium glycocholate and egg albumin were the only combination tested which produced a stable and uniform emulsion of the ester. When 1 cc. of sodium glycocholate solutions of concentrations less than 10 per cent was employed, the total cholesterol content of serial samples indicated that
the ester was not uniformly dispersed. While such preparations could be used to demonstrate the esterase qualitatively, they were obviously unsuitable for quantitative serial studies. On the basis of these findings 1 cc. of 10 per cent sodium glycocholate was adopted as an essential part of the substrate mixture. It should be emphasized that the results obtained with this substrate mixture only define the activity of the enzyme in the presence of this concentration of sodium glycocholate. Sperry and Stoyanoff (9) have shown that the effect of sodium glycocholate on cholesterol esterase varies with the concentration of bile salt and the source of the enzyme. They concluded that the correct explanation for the effects of bile salts must await further experimentation. Thus, while the sodium glycocholate functioned as an emulsifying agent in the preparation of the substrate mixture, it is possible that during incubation it had a secondary effect on the activity of the enzyme. However, this secondary effect, if present, was a constant in all of the experiments reported below.

**Table II**

_Determination of Inactivation Temperature of Cholesterol Esterase of Pancreatin_

The digests were prepared as described in the text. Cholesterol oleate, 100 mg., pH 6.6. Incubation time, 24 hours. Portions of a 20 per cent pancreatin solution were heated in a water bath for 1 hour at the temperatures designated. 1 cc. of heated solutions added to digest.

<table>
<thead>
<tr>
<th>Digest No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature, °C</td>
<td>37</td>
<td>50</td>
<td>55</td>
<td>60</td>
<td>65</td>
<td>70</td>
</tr>
<tr>
<td>Hydrolysis, %</td>
<td>37.7</td>
<td>30.0</td>
<td>17.7</td>
<td>11.3</td>
<td>0.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

In preliminary experiments with the substrate mixture, control digests containing 1 cc. of water or of boiled pancreatin solution were incubated concurrently with active digests. These consistently showed no hydrolysis and were omitted in later experiments.

_Characterization of Hydrolytic Cholesterol Esterase of Pancreatin—_The pancreatin, U. S. P., Merck, was suspended in a glycerol-water mixture (1:1) to give a 20 per cent concentration, except in Experiment IV on the effect of the concentration of enzyme.

**Experiment I; Inactivation Temperature—_Shope (10) reported that the temperature at which the enzyme is inactivated lies somewhere below 67°, and that boiling for 5 minutes completely destroyed the activity. Sperry and Stoyanoff (9) incubated serum for 1 hour at 55–60° to inactivate the enzymes before using it as substrate. In the present experiment portions of the 20 per cent pancreatin suspension were heated for 1 hour at the temperatures designated in Table II, and then 1 cc. samples were added to the substrate mixture and incubated as usual. The data in Table II
show that the enzyme responsible for the hydrolytic activity was inactivated by heating for 1 hour at 65°. In several experiments not reported here, the activity was completely destroyed by placing the pancreatin solution in a boiling water bath for 15 minutes.

**Experiment II; Determination of Optimum pH for Hydrolytic Activity**—Using serum as a substrate, Klein (11) reported two types of hydrolyzing cholesterol esterases. One type found in liver, spleen, kidney, and intestinal mucosa had an optimum pH of 5.3. The other was found in pancreas and was active over a pH range of 5.6 to 9.2, with an optimum range from pH 6.3 to 8.7. Recently, Le Breton and Pantaleon (12) reported that dog pancreas was active at pH 5.3 and at 7.3.

### Table III

*Influence of pH on Hydrolysis of Cholesterol Oleate by Pancreatin*

The digests were prepared as described in the text. Cholesterol oleate, 100 mg. Enzyme, 1 cc. of 20 per cent pancreatin. Time, 24 hours.

<table>
<thead>
<tr>
<th>Digest No.</th>
<th>pH</th>
<th>Hydrolysis (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0*</td>
<td>3.35</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>5.25</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>5.33</td>
</tr>
<tr>
<td>4</td>
<td>6.0</td>
<td>5.73</td>
</tr>
<tr>
<td>5</td>
<td>7.0</td>
<td>6.64</td>
</tr>
<tr>
<td>6</td>
<td>8.0</td>
<td>7.11</td>
</tr>
<tr>
<td>7</td>
<td>9.0</td>
<td>7.35</td>
</tr>
<tr>
<td>8</td>
<td>9.0*</td>
<td>9.60</td>
</tr>
</tbody>
</table>

* See Experiment II in the text.

A series of potassium phosphate buffers (0.15 M) in intervals of 1 pH unit from pH 4 to 9 was used in preparing the substrate mixtures. For the extreme values hydrochloric acid and sodium hydroxide were added to substrate mixtures containing the buffers of pH 4 and 9 respectively. The data, shown in Table III, indicate that the enzyme has a sharp maximum for hydrolytic activity at approximately pH 6.6. In this experiment and in additional runs there was no indication of a second optimum pH for the hydrolytic activity.

**Experiment III; Time-Rate Relationship and Effect of Substrate Concentration**—Fig. 1 shows the time-rate curves for three concentrations of substrate. The curves for the two higher concentrations approach those for a first order reaction. The curve for 33 mg. of substrate is intermediate between those for zero order and first order reactions. Comparable data
were also obtained with 2.5, 5, and 10 per cent pancreatin solutions. In general the specific reaction rate decreased with time at all substrate and enzyme concentrations studied.

**Experiment IV; Effect of Concentration of Enzyme**—A 20 per cent solution of pancreatin was diluted with a glycerol-water mixture (1:1) to give concentrations of 2.5, 5, and 10 per cent. The results shown in Fig. 2 were obtained with 100 mg. of substrate per digest. The percentage hydrolysis was not strictly proportional to the enzyme concentration over the range studied. There was a decrease in the percentage hydrolysis per unit amount of pancreatin as the concentration of enzyme increased. This is a common finding with crude enzyme preparations as used in these experiments. However, the data suggest that under specific conditions of substrate concentration, pH, and incubation time a quantitative assay of the enzyme activity will be possible.

**SUMMARY**

The preparation of a cholesterol oleate substrate mixture and standard conditions suitable for studying the cholesterol ester-hydrolyzing activity...
of tissues and fluids are described. The substrate mixture was used in studying the following characteristics of the hydrolytic activity of a commercial pancreatin. The enzyme was inactivated by boiling for 15 minutes or heating for 1 hour at 65°. The optimum pH was approximately 6.6. The time-rate relationships during a 24 hour incubation period for a series of substrate and enzyme concentrations were determined.

BIBLIOGRAPHY

1. Kondo, K., Biochem. Z., 26, 243 (1910); 27, 427 (1910).
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