Photometric Procedure for Determining Esterase Activity

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In most of the analytic procedures for assaying the esterase content of biologic tissues and fluids, the extent of enzymatic hydrolysis is determined either titrimetrically, manometrically, or photometrically. In the photometric methods the alcoholic component of the substrate is such that its unesterified form can be measured either directly as such (1–6), or indirectly as a colored derivative (7–15). The introduction of photometry into the study of esterases marked an important advancement in the refinement of methods. Nevertheless, some of the photometric procedures possess undesirable features. Thus, the primary objective of the study reported herein was to devise an improved method for determining esterase activity.

EXPERIMENTAL

The technic of chromogenesis used most widely in the methods of indirect measurement is that of coupling the alcoholic component, when phenolic in nature, with a diazonium salt to form an azo dye. The present method employs esters of 2-naphthol as substrates. Chromogenesis is achieved by coupling the 2-naphthol with 4-sulfamoylbenzenediazonium chloride.

The stepwise procedure of the assay is presented in the ensuing section, which is followed by a discussion of some of the chemical and biologic aspects of the method.

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PROCEDURE

Reagents

1. Stock solution of 2-naphthyl ester, $2.96 \times 10^{-4}$ M. To a 10-ml. volumetric flask, add successively 3.75 Gm. of melted Brij 35¹, 0.296 mM. of a 2-naphthyl ester and approximately 5 ml. of redistilled 1, 4-dioxane, reagent grade. Mix the contents of the flask, warming slightly if necessary to obtain solution, and dilute to volume with additional dioxane. The ester should contain no free 2-naphthol or, at the most, only traces. Stock solutions of the caprylate and the palmitate esters are stable for at least several months if refrigerated.

2. Phosphate or citrate buffer, 0.067M. The choice of buffer will depend upon the pH optimum of the esterase.

3. Phosphate buffer, 1.0M, pH 6.8.

4. 4-Sulfamoylbenzenediazonium chloride solution (16). Prepare this reagent by mixing equal volumes of a solution of sulfanilamide, 0.035M in 0.48N hydrochloric acid, and a solution of sodium nitrite, 0.042M. Let this mixture stand for several minutes at room temperature, and then place it in an ice bath. When kept ice-cold, this reagent is stable for at least 6 to 8 hours.

5. Hydrochloric acid, 1.6N.

6. Sodium hydroxide, 3.3N.

Assay Technic

Prepare a buffered solution of substrate in the following manner immediately prior to its use. All reagents should be at room temperature. Into a gently agitated mixture of 40 ml. of 0.067M phosphate or citrate buffer and approximately 50 ml. of water, slowly add 1 ml. of the stock solution of ester with a pipet, the tip of which is held beneath the surface of the mixture. Dilute to 100 ml. with additional water.

Transfer 5 ml. of the buffered solution of substrate to a 25- x 200-mm. Pyrex test tube calibrated to contain 25 ml. Place the tube in a constant-temperature water bath. After equalization of temperature, add 1 ml. of an esterase preparation, and mix the contents of the tube thoroughly. Following incubation, the length of which is determined primarily by the rate at which the substrate is hydrolyzed, add 5 ml. of 1.0M phosphate buffer, pH 6.8. Immediately add 0.5 ml. of 4-sulfamoylbenzenediazonium chloride solution. Wait exactly 1 minute for the de-

¹ Brij 35, a polyoxyethylene lauryl alcohol, is a colorless, nonionic surfactant having a melting range of approximately 40-44° and is manufactured by Atlas Powder Company, Wilmington 99, Del.
velopment of color, and add 5 ml. of 1.6N hydrochloric acid. Place the tube in water which is kept at a rolling boil for 20 minutes. Cool the contents of the tube to room temperature. Add 5 ml. of 3.3N sodium hydroxide, letting it flow down the wall of the tube. Without undue agitation, dilute the contents of the tube to 25 ml. with water and mix thoroughly. Measure the optical density of this solution at 460 μ using as a reference blank the solution from a concomitant control in which the esterase was inactivated prior to incubation by heating.

Many esterase preparations may be assayed simultaneously by staggering the addition of enzyme to the buffered solution of substrate at 2-minute intervals. In order that all samples will be incubated for the same length of time, the sequential addition of 1.0M phosphate buffer, 4-sulfamoylbenzenediazonium chloride solution and 1.6N hydrochloric acid, all of which requires approximately 1.5 minutes for a single tube, is staggered also at 2-minute intervals. At this stage, i.e., after addition of the hydrochloric acid, subsequent steps need not be performed immediately because the color is stable if the mixture is not exposed to sunlight.

Standard curve

From stock solutions of 2-naphthol in 1,4-dioxane and Brij 35, prepare buffered solutions of this compound in the same way that the buffered solution of substrate is prepared. Take 5-ml. samples of these buffered solutions, and, except for incubating them with esterase, subject them to the same procedure outlined in the assay technic. When measuring the optical densities of the final solutions, use as a reference blank the solution of a concomitant control without the 2-naphthol. The stock solutions of 2-naphthol are somewhat less stable under refrigeration than are those of the 2-naphthyl esters and, therefore, should be prepared immediately prior to their use. The standard curve is reproducible, thus obviating the preparation of standards for every series of assays.

DISCUSSION

2-Naphthyl caprylate and 2-naphthyl palmitate, the esters used in this study were synthesized according to the general procedure of Nachlas and Seligman (9) and possessed melting points of 43.0–44.0° and 69.0–70.5°, respectively. The effect of pH on the nonenzymatic hydrolysis of these two esters was not studied specifically, but assays have been conducted over the pH range of 4.6 to 7.9 with either no or very little such hydrolysis occurring.

Another method for the synthesis of 2-naphthyl esters has been described by Gomori (7).
Aqueous mixtures of water-insoluble esters frequently have been prepared by dissolving the ester in a water-soluble organic solvent and then introducing a small quantity of this solution into water (3, 7, 8, 9, 11, 14, 17). Such action with 2-naphthyl caprylate and 2-naphthyl palmitate, as well as many other esters, produces an opalescent suspension, which is undesirable for photometric measurement. In the present study it was found that nonopalescent solutions of substrate could be obtained if a sufficient quantity of Brij 35 were added to the mixture of ester and organic solvent. The suggested concentration of Brij 35 in the stock solution, 0.375 Gm. per ml., is in excess of the minimum required to produce nonopalescence. If the buffered solution of substrate is refrigerated, however, precipitation of the ester may occur.

Chromogenesis is accomplished by coupling 4-sulfamoylbenzenediazonium chloride with 2-naphthol to form 1-(4-sulfamoylbenzenediazo)-2-naphthol, an azo dye. The selection of this particular diazonium salt was based on its previous use in the quantitative determination of 2-naphthol by Harfenist and Baltzly, who were studying the acid-catalyzed alcoholysis of 2-naphthyl esters. Inasmuch as the reactants are the diazonium ion and the naphthoxide ion, not the undissociated naphthol molecule (18), the velocity of coupling increases as the hydrogen ion concentration of the reacting medium decreases. The purpose, therefore, of adding the 1.0M phosphate buffer having a pH of 6.8 immediately prior to the diazonium salt is to provide a hydrogen ion concentration that is low enough to facilitate rapid formation of the azo dye yet high enough to prevent nonenzymatic hydrolysis of the substrate. The procedure of Harfenist and Baltzly utilizes a solution of sodium acetate instead of a phosphate buffer. The phosphate buffer was selected for the assay described herein because of its greater buffering capacity in the region of pH 6.6-6.8. The quantity of phosphate buffer used, 5 ml., is sufficient to minimize pH changes that are induced either by the buffer employed during incubation or by the solution of diazoniun salt. Dye formation, for which 1 minute is allotted, reaches completion between 15 and 30 seconds after the diazonium salt is added.

One half milliliter of the solution of diazonium salt is equivalent to six times the quantity of ester present in 5 ml. of the buffered solution of substrate. Thus, the excess diazonium salt must be destroyed to prevent it from coupling with any 2-naphthol that may be liberated subsequently by nonenzymatic hydrolysis, e.g., that caused by the addition of sodium hydroxide. Harfenist and Baltzly effected this destruction immediately after dye formation by heating the solution to its boiling point, which
action converts the excess diazonium salt to 4-hydroxybenzenesulfonamide (18), a colorless compound (19). In the present study such heating, even in the absence of 2-naphthol, produced an intense yellow color, which is believed to be an additional azo dye formed by some of the diazonium salt coupling with its own degradation product. This difficulty was circumvented by lowering the pH to approximately 1.4 with hydrochloric acid after formation of the naphthyl azo dye. The excess salt then can be destroyed by heating without encountering the undesirable side reaction because the coupling of diazonium salts with phenolic compounds does not occur in a strongly acid solution (18). The naphthyl azo dye is unaffected by such heating.

The naphthyl azo dye, although some of it may be held in suspension by the Brij 35, is insoluble in acid but is soluble in a strongly alkaline medium. Thus, sodium hydroxide is added, whereupon the dye changes in color from yellow-orange to red-orange. The color change, which suggests that the hydroxy radical of the dye undergoes ionization, requires approximately 4.5 ml. of 3.3N sodium hydroxide to complete. The relative optical density of the dye as a function of wavelength is illustrated in Fig. 1. The optical density at 460 mμ is directly proportional to the concentration of 2-naphthol over the range of 0 to 240 μg. per 25 ml. (Fig. 2). The stability of the dye is excellent provided that exposure to direct sunlight, which causes fading, is avoided. Solutions held under somewhat subdued lighting for as long as 24 hours decreased only slightly in optical density.

Inasmuch as several nonionic surfactants have been reported to reduce the activity of pancreatic lipase (20), investigation of the effect of Brij 35

![Fig. 1. Relative optical density of 1-(4-sulfamoylbenzenesazo)-2-naphthol as a function of wavelength.](image)
on the rate at which 2-naphthyl caprylate and 2-naphthyl palmitate are hydrolyzed by different esterases was deemed advisable. Stock solutions of ester were prepared such that the buffered solutions of substrate varied in concentration of Brij 35 (75 to 525 mg./100 ml.) but contained equal levels of all other components. The sources of esterase were aqueous extracts of the liver of swine and pharyngeal tissue of young calves. The results of these determinations are presented in Fig 3, in which the rates of hydrolysis are expressed in arbitrary units, the maxima being assigned the value of 100 and all others adjusted in proportion thereto. These data clearly indicate the Brij 35 may have either a stimulatory or an inhibitory effect on the rate of enzymatic hydrolysis. The concentration for maximum activity depends markedly on the substrate and, to a lesser extent, on the source of esterase. The rate of enzymatic hydrolysis also is affected by 1,4-dioxane. When present in the buffered solution of substrate at the level of 5 to 10 per cent, this solvent reduces esterase activity but does so to a lesser extent than the same concentration of acetone. Thus, 1,4-dioxane was selected for the procedure described herein, and its concentration was reduced to approximately that required to liquefy the stock solution of ester.
The effect of enzyme concentration on the rate at which 2-naphthyl caprylate is hydrolyzed is illustrated in Fig. 4. The source of esterase was the pharyngeal tissue mentioned previously. Only the initial rates of hydrolysis appear to be directly proportional to enzyme concentration.

This procedure is suited for assaying blood serum as well as tissue extracts. The results from such determinations are presented in Table 1. One milliliter of a fifty-fold dilution of serum was used per assay. Determinations were made in triplicate to illustrate the precision of the method. In one set of the triplicate samples, a flocculant precipitate was noted following the heating in boiling water. This precipitate, presumably protein, went into solution upon addition of the sodium hydroxide and caused no interference in the determination.

The procedure described is believed to have several advantages over certain other photometric esterase procedures. First, it does not require a photometer capable of measuring light absorption in the ultraviolet region of the spectrum as do the methods of Zeller and Hofstee (3–5). A second advantage is that the 2-naphthyl esters do not appear to undergo non-enzymatic hydrolysis as readily as the substrates reported in some methods (1–5, 12). The solubility of the naphthyl azo dye in a strongly alkaline medium also is advantageous in that it eliminates the necessity of having to extract the dye with an organic solvent (9–11) or of having to hold it in colloidal suspension with a surfactant (7). In other azo dye methods (7, 9–11), with the exception of one (14), no adjustment in pH is made immediately prior to dye formation, the rate of which is dependent upon
Fig. 4. Progressive hydrolysis of 2-naphthyl caprylate at pH 6.5 by the esterase from various quantities (0.2, 0.4, 0.6, 0.8, and 1.0 mg.) of an acetone powder of calf pharyngeal tissue.

Table 1. Hydrolysis of 2-Naphthyl Caprylate by the Esterase of Blood Serum at pH 7.0

<table>
<thead>
<tr>
<th>Source of serum</th>
<th>Age of animal</th>
<th>Triplicate determinations (% hydrolysis)</th>
</tr>
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<tbody>
<tr>
<td>Calf</td>
<td>1½ weeks</td>
<td>0.8</td>
</tr>
<tr>
<td>Calf</td>
<td>1½ weeks</td>
<td>1.6</td>
</tr>
<tr>
<td>Calf</td>
<td>8 weeks</td>
<td>6.7</td>
</tr>
<tr>
<td>Calf</td>
<td>8 months</td>
<td>11.7</td>
</tr>
<tr>
<td>Calf</td>
<td>8 months</td>
<td>10.2</td>
</tr>
<tr>
<td>Calf</td>
<td>8 months</td>
<td>8.1</td>
</tr>
<tr>
<td>Lamb</td>
<td>6 months</td>
<td>17.9</td>
</tr>
<tr>
<td>Lamb</td>
<td>8 months</td>
<td>28.6</td>
</tr>
<tr>
<td>Lamb</td>
<td>9 months</td>
<td>24.6</td>
</tr>
<tr>
<td>Lamb</td>
<td>9 months</td>
<td>25.3</td>
</tr>
</tbody>
</table>

pH. Thus, the flexibility of these procedures (7, 9–11) is reduced in that the pH of incubation may be too low to permit complete formation of the dye. Such is not the case for the procedure described inasmuch as the pH is adjusted with 1.0M phosphate buffer. Nor is the pH of the final solution nearly so critical as it is in the method of Huggins and Lapides as emphasized by Dirks and Boyer. The use of Brij 35 to prepare non-opalescent mixtures of the substrate also is believed to be advantageous, particularly in view of the fact that many esters are very insoluble in water.
The manner in which Brij 35 modifies the rate of enzymatic hydrolysis, on the other hand, reduces somewhat the flexibility of this method for studying either the specificity of a single esterase for different substrates or the rates at which a single substrate is hydrolyzed by different esterases. Nevertheless, the importance of studying the influence of stabilizing agents on enzyme activity is emphasized by the variable effect of Brij 35. Two other factors also may limit the flexibility of the present method. The first of these is the presence of excessive quantities of protein or particulate matter in the enzyme extract causing opalescence or a precipitate in the final solution. Thus far, such difficulty has not been encountered, although efforts have been made to clarify the enzyme extracts as much as possible either by centrifugation, or by freezing and thawing as suggested by Gomori (7). The second problem is the natural occurrence of compounds in tissues and fluids that will couple with the diazonium salt to yield a colored derivative. To date such compounds have been found in excessive quantities only in rumen fluid.

SUMMARY

A procedure for determining the esterase content of biologic tissues and fluids has been presented. Fatty acid esters of 2-naphthol are employed as substrates. The 2-naphthol liberated by hydrolysis is coupled with a diazoniunm salt to form an azo dye, the concentration of which is estimated photometrically. Distinctive features of this method are (1) the use of Brij 35 to prepare nonopalescent solutions of substrate, (2) the adjustment of pH to ensure complete formation of the azo dye, and (3) the solubilizing of the azo dye by adding sodium hydroxide. This assay procedure is sensitive to very small amounts of esterase, and the stability of its color is excellent if proper precautions are taken.

REFERENCES