Similar results were obtained with samples of acids when as low as approximately 10 mg of total acids were used for esterification. Although results in Table 1 are for saturated acids only, recovery values ranging from 97 to 101% have been obtained for oleic and linoleic acids. Results on 20 samples prepared and analyzed similarly to that represented by Table 1 exhibit a pooled error mean square of 3.67.

The technique used here can be applied to the methyl transesterification of triglycerides. Times and temperatures recommended by Metcalf and Schmitz (13) have given us good results in this regard.

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References

Apparent Homogeneity of Lactoperoxidase in Gel Electrophoretograms

Lactoperoxidase (doner: H₂O₂ oxidoreductase, EC 1.11.1.7), a heme-containing protein which catalyzes the transfer of oxygen from peroxides to other substances, is one of the principal enzymes found in cows' milk. Polis and Shmukler (4) isolated the enzyme from whey by salt fractionation in alkaline solutions and displacement chromatography on columns of calcium phosphate and silica-calcite. A molecular weight (Mₖ) of 82,000 was reported for the crystallized enzyme. Moving-boundary experiments revealed the presence of two electrophoretically distinct species, designated A and B. Detailed spectral, electrophoretic, and
kinetic studies performed on these two enzyme forms caused the authors to conclude that lactoperoxidase A was the native form and form B emanated from A, induced by treatments encountered during isolation. Morrison and Hultquist (2), employing cation exchange resins, obtained the enzyme in a single form in at least four isolations, and two forms were obtained at other times. Groves (1), while working with the red protein encountered lactoperoxidase in his disc electrophoretograms. The poor resolution observed at pH 9.5 was improved in gels at pH 4.3. A large portion of the lactoperoxidase activity was identified with the fast-moving, principal protein zone, but lesser activities were identified with the smaller, slow-moving bands.

Most of the available information on lactoperoxidase indicates that the enzyme exists in nature as a single molecular species and that process-induced alterations in the native protein might possibly account for the observation of other forms. Yet, the question concerning the existence of isozymes is unanswered and will remain so until an extensive study is undertaken on the milk of numerous individuals. It is of interest in this regard that Ockerse et al. (3) demonstrated the presence of at least seven peroxidase isozymes in the stem sections of the dwarf pea. However, the ease for genetic variant or isozyme forms of lactoperoxidase is weakened by the observation of Morrison and Hultquist (2), that a single form of the enzyme was isolated from sources of pooled milk obtained from two widely separated geographical locations.

The purpose of this experiment was to determine if more than one form of lactoperoxidase could be detected in pooled milk by methods designed to bring the enzyme to the point of characterization with a minimum of manipulation.

A lactoperoxidase-rich whey fraction was isolated from freshly drawn, uncooled cows' milk, essentially as outlined in Figure 1. These protein-containing fractions were submitted to zonal electrophoresis in Smithies-type starch gels, prepared by adding 25 g of hydrolyzed starch (Connaught) to sodium hydroxide (0.028 M)-formic acid (0.05 M) buffer, pH 3.8. The gel was formed on a horizontal, water-cooled bed and connected to the electrode-buffer vessels by filter-paper wicks (E-D no. 652). The initial current applied to the gel was 5.3 mA/cm² of cross-sectional area which decreased slowly during electrophoresis (~16 hr) to ~3.0 mA/cm².

Upon completion of electrophoresis, the gel was sliced horizontally and separated into three sections. One section was flooded with a solution of guaiacol (5 × 10⁻⁸ M) and hydrogen peroxide (5 × 10⁻³ M) in 0.2 M phosphate buffer, pH 5.8; lactoperoxidase-containing zones developed an orange color. A second section was treated with Amido Black to reveal protein zones. Corresponding slots from these sections are shown in Figure 2. Of primary significance is the observation that only one peroxidase-
positive zone could be detected. This was also true for gels run under alkaline conditions and at high voltages, as employed by Ockerse et al. (3) in their studies with plant peroxidases. Interestingly, the enzyme was active following electrophoresis in gels containing up to 3 M urea and still showed but one zone. Results of these studies suggest that lactoperoxidase exists as a single molecular species.

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Improved Method for Detection of Microbial Lipolysis

Many of the proposed plating media for the detection of microbial lipolysis have been based on the dye indicator technique of Turner (10) or the clearing zone technique of Anderson (2). In comprehensive studies of the different media, Jensen and Grcttie (5), and more recently Rath (8) were unable to find any of them entirely satisfactory. The major criticisms were: difficulty in interpreting the result, particularly in cases of low lipolytic activity; toxicity of the dyes and some of the fats at the levels employed; and some of the dyes used are oxidation-reduction indicators rather than indicators of fat hydrolysis.

In an effort to minimize some of these difficulties, Rath (8) devised a double-layered agar technique. The basic principle in this method is the same as in the now-classical tallow layer medium of Eijkman (3). One of the layers is designed for the growth of the organisms, whereas the other is prepared solely for the detection of lipolytic activity. Similar methods have recently been employed by Alford and Steinle (1) and Fryer, Lawrence and Reiter (5), who also modified the clearing zone technique, using thin layers of tributyrin agar on microscope slides. Cell suspensions were placed in agar wells and upon diffusion of the enzyme through the agar the hydrolyzed tributyrin gave rise to clear, transparent zones around the wells.

In spite of the rather extensive literature on plating techniques for the detection of microbial lipolysis, little information was available on how factors like the triglyceride or fat concentration in the medium, depth of agar layer, agar well size, concentration of plated cell suspension, and incubation time would influence the sensitivity and usefulness of such plating techniques. The objective of this report is to show how these factors affect fat hydrolysis, using a modified agar well assay technique.

Experimental Procedure

1. Preparation of tributyrin agar plates. The basal medium consisted of MRS broth (Difco) with addition of 1.5% w/v Bacto agar. After autoclaving at 121 C for 15 min, the medium was cooled to 60 C. Filter-sterilized tributyrin was then added and the mixture emulsified with an ultrasonic disintegrator (Bronson Sonifier Model S125) at 8 amp for 1.5 min. Immediately after sonification the medium was distributed in 1-mm layers in flat-bottomed petri dishes and allowed to harden on a leveled surface.

The effect of agar depth on zone size was studied in a preliminary experiment, using 1-, 2-, and 3-mm layers of agar medium. The choice of 1-mm agar depth, which will be discussed later, was based on information from this experiment.

2. Punching wells in the agar. On each plate wells of 3-, 5-, and 7-mm diameter were conveniently punched in the agar medium by connecting specially made metal tubing to a vacuum line and applying a very slight vacuum. The wells were evenly spaced in a triangular pattern, using a paper template placed beneath the agar dish.

3. Preparation of cell suspension. The organism used in this study was Lactobacillus brevis strain X2. Twelve-hour-old cultures grown on regular MRS broth at 30 C were used to inoculate MRS broth containing 0.05% glycerol and 1% half-and-half. The glycerol was filter-sterilized and the half-and-half autoclaved at 121 C for 15 min before addition to sterilized broth. The inoculated medium was then incubated for 24 hr at 30 C. The organisms were harvested by centrifugation at 10,000 × g for 10 min and the cell pellet resuspended in a small volume of 0.01 M NH₄OH-NH₄Cl buffer pH 7.2. The final concentration was approxi-