Long Chain Base-Acetyl Coenzyme A Acetyltransferase from the Microsomes of *Hansenula ciferri*

I. ISOLATION AND PROPERTIES*

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**SUMMARY**

Microsomes of the yeast, *Hansenula ciferri* NRRL Y-1031, F-60-10, contain an enzyme (long chain base-acetyl coenzyme A acetyltransferase, EC 2.3.1.1) which catalyzes the transfer of the acetyl moiety of acetyl coenzyme A to both the amino and hydroxyl groups of the sphingosine bases; a separation of these two transfer reactions could not be achieved. The enzyme also catalyzed an acetyl transfer to the hydroxyl groups of N-acetylated sphingosine bases and to the amino groups of normal aliphatic primary amines of 6 to 18 carbon atoms, but not to compounds such as p-nitroaniline, glucosamine, secondary amines, or primary alcohols. A similar enzyme was not found in rat organs nor in extracts of pigeon liver acetone powder.

The reaction rates were directly proportional to enzyme concentration and time of incubation. The optimal pH depended on the type of buffer used. The reaction rates could be correlated to the charge conferred upon the micelles of the bases by the various buffers. The reaction was not reversible and an exchange between the products and substrates could not be shown. It was inhibited by coenzyme A but not by the lipid products.

Several strains of the yeast Hansenula ciferri produce large quantities of sphingosine bases which are secreted into the growth medium (1). In a previous investigation (2), 25 strains of the genus *Hansenula* were compared and classified on the basis of the quantities of the LCB's produced. These ranged from 120 to 266 pmol of LCB's per liter of growth medium (“high producers”) to 2 to 5 pmol per liter (“low producers”). These compounds (mostly phytosphingosine) occur not as free, but as partially or fully acetylated bases (1). The enzyme responsible for this acetylation was identified and some of its properties were the subject of a preliminary communication (3). This enzyme utilized acetyl coenzyme A for the transfer of the acetyl group to the LCB’s. It was of interest that this enzyme was present in the high producers but was practically absent from those yeast strains (low producers) which produced small quantities of the bases. The implication of this to the “diseased state” of the yeast was discussed (2).

Several acetyltransferases are known and were classified according to the donors and acceptors of the acetyl group. The acceptor might be a hydroxyl or an amino group, but no enzyme has been described to date which transfers the acetyl group to both amino and hydroxyl groups. Also, none of the acetyltransferases utilized a sphingosine base as an acceptor of the acetyl group. The sphingosine bases are acylated at their amino groups by a long chain fatty acid (4, 5) or long chain fatty acyl coenzyme A (6, 7). This paper further describes the properties of the enzyme, present in the microsomes of *H. ciferri*, which transfers the acetyl group of acetyl-CoA to both the amino and hydroxyl groups of the sphingosine bases or to the amino group of long chain primary amines.

**EXPERIMENTAL PROCEDURE**

**Materials**

Acetyl and butyryl coenzyme A as well as [2-3H]acetyl coenzyme A, were synthesized with the respective anhydrides for acylation of the free coenzyme (8); these acyl coenzyme A derivatives contained no more than 1.5% of free coenzyme A and less than 0.1% of acetic or butyric acid, respectively.

Sphingosine, dihydrosphingosine, and phytosphingosine were prepared according to the method of Barenholz and Gatt (9). N-Acetyl phytosphingosine was prepared by mild alkaline hydrolysis of the acetylated bases, isolated from the growth medium of *H. ciferri*. It was further purified with Silica Gel S columns (Riedel de Haen) from which it was eluted with 5% methanol in chloroform. Its purity was tested by thin layer silica gel chromatography, using chloroform methanol, 9:1 (10), and by gas-liquid chromatography of the trimethylsilyl derivative (11). N-Acetyl dihydrosphingosine and N-acetyl sphingosine were prepared according to the procedure of Gatt (4) or Carter and Gaver (11). N-Acetyl hexadecylamine and N-butyryl dihydrosphingosine were prepared according to the...
method of Carter and Gaver (11), using acetic or butyric anhydrides, respectively. Tetraacctyl phytyosphingosine was isolated from the growth medium of H. ciferri and purified on a silica acid (Mallincrodt) column, from which it was eluted with 2% methanol in chloroform. It was characterized on thin layer silica gel plates, with chloroform-methanol, 30:1. DL-Erythro sphingosine, DL-erythro dihydroxyphosphatidyl glycerol, dN-acetyl dichloro-

\[ \text{In an alternative procedure, 1.2 ml of ether were added to the hydrolisy mixture; after stirring, the ether phase was collected and the aqueous methanolic phase was again shaken with 1.2 ml of ether. The two ethereal extracts were combined, backwashed with 1 ml of water, evaporated, and counted as above.}

One unit of activity is defined as a nanomole of [3H]acetate transferred or a nanomole of coenzyme A released during the transfer reaction.

RES ok

Preparation of Enzyme

The yeast was cultured and disrupted according to the method of Braun and Snell (14). Care was taken to keep the growth temperature at no more than 27°C. After harvesting, the packed cells were suspended in 0.05 M potassium phosphate, pH 7.0, which contained 20% glycerol (Buffer A) and frozen at -20°C. The cells were disrupted within 24 to 40 hours and microsomes were prepared (3). In some experiments the 14,000 × g supernatant was centrifuged for 65 min at 290,000 × g, the sediment was washed in Buffer A and recentrifuged at moderate speed. Either microsomal preparation retained its acetyltransferase activity for at least 18 months at -20°C. If suspended in phosphate buffer (0.05 M, pH 7.0) without glycerol, the enzymatic activity was lost within 1 week or less when stored at -20°C. Enzymes were added to solubilize the microsomes with detergents, proteases, lipases and phospholipases, and sonic or ultrasonic irradiation. None of these resulted in the formation of a soluble enzyme.

Subcellular Localization of Enzymatic Activity

Yeast cultures were grown, harvested, and disrupted as described elsewhere (14). The extract was separated into the following fractions: unbroken cells and debris were removed by two successive centrifugations at 800 and 1200 × g for 10 min each.

The supernatant was centrifuged at 10,000 × g for 25 min and the sedimented mitochondria were suspended in Buffer A (0.5 ml per g of packed cells). Microsomes were then sedimented for 3 hours at 100,000 × g, the supernatant was retained and the microsomal segment was suspended as above.

Each of the fractions was assayed for both N- and O-acetylation as described under "Methods." The results were calculated in terms of "relative specific activity" (Table I). The

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Relative specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetylation of phytyosphingosine</td>
<td>O- and N-acetylation of phytyosphingosine</td>
</tr>
<tr>
<td>1,300 × g supernatant</td>
<td>1.0</td>
</tr>
<tr>
<td>10,000 × g supernatant</td>
<td>1.8</td>
</tr>
<tr>
<td>100,000 × g supernatant</td>
<td>5.0</td>
</tr>
<tr>
<td>100,000 × g supernatant</td>
<td>0</td>
</tr>
</tbody>
</table>

* Relative specific activity is defined as the percentage of total activity divided by the percentage of total protein, in each fraction (15).
Fig. 1. Effect of pH and type of buffer on the reaction rates. Incubation mixtures were prepared and the products were determined as described under “Methods.” Microsomal protein, 0.2 mg, and 20 μmoles of each of the following buffers were used: potassium acetate, pH 4.0 to 5.6; potassium phosphate, pH 5.8 to 8.0 (solid lines); potassium borate, pH 7.6 to 10.7 (dashed lines). Incubation time, 60 min. ■ hexadecylamine (N-acetylation); ● O, phytosphingosine (N-acetylation); □ S, phytosphingosine (N- and O-acetylation), ○ N-acetyl phytosphingosine (O-acetylation).

Table II shows the electrophoretic mobility of hexadecylamine and dihydrosphingosine at certain pH values of the above three buffers. The complete incubation mixture contained, in a volume of 0.4 ml, 0.8 mg of microsomal protein, 0.1 μ mole of dithiothreitol, 0.4 μ mole of N-acetyl phytosphingosine, 0.05 μ mole of CoA, 0.5 μ mole of ATP, 0.1 μ mole of [2-3H]acetate (sodium salt, 8 × 10^6 dpm per μ mole), 1 μ mole of MgCl₂, and 40 μ moles of potassium phosphate buffer, pH 7.8. When [2-3H]acetate CoA replaced the mixture of acetate, ATP, and CoA, 0.2 μ mole of this compound (6.4 × 10^6 dpm per μ mole) was used. After 90 min at 37° the products were determined as described under “Methods.”

Table II

<table>
<thead>
<tr>
<th>Buffer solution</th>
<th>Dihydrosphingosine</th>
<th>Hexadecylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium acetate, pH 5.0</td>
<td>+11.00</td>
<td>+12.30</td>
</tr>
<tr>
<td>Potassium phosphate, pH 7.9</td>
<td>+3.85</td>
<td>-1.42</td>
</tr>
<tr>
<td>Potassium borate, pH 9.3</td>
<td>-1.83</td>
<td>+4.02</td>
</tr>
</tbody>
</table>

* Mobility units = μsec⁻¹ volt⁻¹ cm⁻¹.

**Effect of Detergents**

The effects of several detergents were tested on either N- or O-acetylation. The concentrations used were 0.025 to 0.2% of sodium lauryl sulfate and 0.05 to 0.5% of Cutexum, Triton X-100, and the sodium salts of cholic, deoxycholic, or taurocholic acids. Various degrees of inhibition were obtained depending on the type and concentration of the detergent. In no case was the enzymatic activity increased by the addition of these compounds.

**Properties of Reaction**

Effect of Protein Concentration and Reaction Time—The rates of N- or O-acetylation of phytosphingosine, O-acetylation of N-acetyl phytosphingosine, or N-acetylation of hexadecylamine, were directly proportional to enzyme concentration (up to at least 1 mg of microsomal protein) and to the reaction time (for at least 2 hours).

Effect of pH and Buffers—Fig. 1 shows the effect of three buffers at pH values between 4 to 10 on the rates of N-acetylation of hexadecylamine, N- and O-acetylation of phytosphingosine, and O-acetylation of N-acetyl phytosphingosine. None of the substrates used were acetylated with acetate buffer at pH 4 to 5.8. In phosphate buffer (at pH 5.6 to 8.0), reaction rates increased, reaching near maximal values at pH 8. When borate replaced this buffer, reaction rates at pH 8 were lower than those obtained with phosphate at the same pH. The rates of N- or O-acetylation of phytosphingosine or N-acetyl phytosphingosine increased with increasing pH of the borate buffer, reaching a maximum above pH 9; the N-acetylation of hexadecylamine was practically constant in this buffer between pH 7.6 and 9.3.
Table IV

Acetylation of sphingosine bases and their derivatives

Table V

Stoichiometry of reaction

and ATP could no longer replace acetyl-CoA as substrate. Butyryl-CoA was neither a substrate for the reaction nor an inhibitor for acetyl group transfer from acetyl-CoA.

Table IV shows an acetyl transfer to several sphingosine bases and derivatives. Only the free bases or their N-acetylated derivatives were substrates for the enzyme. The N-butyryl, N-octanoyl, N-palmitoyl, 1-O-galactosyl, or N-acetyldichloro derivatives of the bases were not acetylated.

Normal aliphatic amines of 6 to 18 carbon atoms (but not secondary amines, such as didecyl- or didodecylamine) were also acceptors of the acetyl groups of acetyl-CoA (Fig. 2). In this experiment, where the concentration of each of the amines was 1 mM, maximal reaction rates were obtained with C₁₆ to C₁₈ amines (Fig. 2).

The following compounds were neither substrates nor inhibitors of the N- or O-acetylation of sphingosine bases or of the N-acetylation of hexadecylamine: phosphatidylethanolamine, glucosamine, p-nitroaniline; normal primary aliphatic alcohols of 10 to 18 carbon atoms, 1-acylglycero-3-phosphorylcholine (lysolecithin).

Stoichiometry of Reaction. Table V shows the stoichiometric relationships between the coenzyme A released and the acetyl groups bound. Using three substrates this ratio was about 1.1 to 1.3.

Reversibility of Reaction—Experiments were performed to test whether the reaction was reversible. N-Acetylated sphingosine or hexadecylamine were incubated with CoA at pH values of 4.5 to 9.5. Neither CoA disappearance nor appearance of free amino groups (16) could be detected. Addition of free LCB's to the reaction mixtures did not change the above results.

In a separate experiment the possibility of an acetyl group transfer from N-acetyl hexadecylamine to dihydrosphingosine was tested; no such reaction was observed. Negative results were also obtained when trials were made to obtain exchange reactions between the acetyl groups of acetyl-CoA and that of N-acetyl hexadecylamine, or transfer of acetyl groups esterified at the hydroxyls of the LCB's to the amino groups of free sphingosine bases. Neither was there any exchange between the acetyl groups bound to the hydroxyls or amino group of the same base molecule.

Inhibition of Reaction—The lipid products of the reaction (i.e. acetylated bases) had no effect on the acetylation, either of the same base or of the other LCB's. Coenzyme A, the second product of the reaction, inhibited both N- and O-acetylation. At 1 mM each, of CoA, LCB, and [2-3H]acetyl-CoA, reaction rates were only 15 to 35% of those obtained in the absence of the CoA. DTNB or p-hydroxymercuribenzoate (each at a concentration of 1 mM) relieved this inhibition by binding the CoA. The inhibition was relieved even when these reagents were added after mixing the enzyme and CoA, thus suggesting that the inhibition by the coenzyme could be reversed. Addition of the two reagents to reaction mixtures containing LCB and acetyl-CoA (but no free CoA) increased reaction rates about 50%. Kinetic experiments of CoA inhibition are presented in the following paper (17). Other thiol compounds, such as dithiothreitol and mercaptoethanol (each at 1 mM) had no effect on reaction rates; this suggests a specific, inhibitory effect of coenzyme A.
The 0- and N-acetylation was tested. These included several detergents (Triton X-100, 0.5 to 5$&; sodium cholate, deoxy-

Effect of Heating-Heating for 10 min at 60$^\circ$ (not shown in the figures), resulted in complete inactivation of both activities. Fig. 3 shows the effect of heating at 47.5$^\circ$. After half a minute, the decrease of the N- and O-acetylations were 34 and 38%, respectively. After 5 min, about 40% of either activity remained.

Trials to Separate N- and O-Acetylation

Numerous experiments were conducted with the purpose of separating the microsomal preparation into two separate enzymes (or activities), one catalyzing an acetyl transfer to the amino and the other to the hydroxyl groups of the LCB’s. These experiments are categorized as follows.

Physical and Chemical Separation—This included treatment with 1 m KCl, buffers at a pH range of 4 to 10, I-butanol, and acetone, proteases (trypsin or chymotrypsin), phospholipases A or C, pancreatic lipase, or various detergents. Microsomal suspensions were also subjected to sonic irradiation at either 10 or 20 kc. After each of the above treatments, the suspensions were centrifuged for 30 min at 40,000 × g, the supernatants were retained and the sediments were dispersed in Buffer A. In most cases the activity was recovered in the sediments and the ratio of N- to O-acetylation was identical with that of the original microsomes. The use of the proteases or acetone resulted in a complete loss of the enzymatic activity. Treatment with SO/cm resulted in recoveries of 20, 5, and 5%, respectively, in the 1.2 × 10$^5$ g × min supernatant.

Kinetic Experiments—Fig. 4 describes two parallel experiments. Experiment 1, in the experiment presented by the solid line also contained 0.2 pmole of N-acetyl phytosphingosine and varying concentrations (0 to 0.5 mM) of hexadecylamine. The second set (dashed line) contained 0.1 pmole of hexadecylamine and varying concentrations (0 to 1 mM) of N-acetyl phytosphingosine. After 120 min at 37$^\circ$, the reaction was terminated and the total acetylation (i.e. the CoA released) was measured as described under “Methods.”

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration (mM)</th>
<th>O-Acetylation of N-acetyl phytosphingosine (a)</th>
<th>N-Acetylation of phytosphingosine (b)</th>
<th>N-Acetylation of hexadecylamine (c)</th>
<th>Ratio a:b</th>
<th>Ratio a:c</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0 around</td>
<td>5.5</td>
<td>3.5</td>
<td>2.2</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>CoA</td>
<td>0.5</td>
<td>1.1</td>
<td>0.5</td>
<td>0.4</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td>DTT</td>
<td>1.0</td>
<td>5.7</td>
<td>3.4</td>
<td>2.4</td>
<td>1.7</td>
<td>2.4</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>1.0</td>
<td>4.8</td>
<td>3.0</td>
<td>2.0</td>
<td>1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>1.0</td>
<td>9.9</td>
<td>5.6</td>
<td>3.3</td>
<td>1.6</td>
<td>2.7</td>
</tr>
<tr>
<td>DTNB</td>
<td>1.0</td>
<td>6.6</td>
<td>3.3</td>
<td>2.4</td>
<td>2.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

The ratio of N- to O-acetylation remained practically constant with all agents. Table VI shows the effect of several thiols and -SH inhibitors. The effects of these compounds varied. Thus coenzyme A inhibited either reaction while p-hydroxymercuribenzoate increased the reaction rates. However, the ratio of N- to O-acetylation remained practically constant with all agents.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration (mM)</th>
<th>O-Acetylation of N-acetyl phytosphingosine (a)</th>
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</tr>
<tr>
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<td>5.7</td>
<td>3.4</td>
<td>2.4</td>
<td>1.7</td>
<td>2.4</td>
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<td>Mercaptoethanol</td>
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<td>4.8</td>
<td>3.0</td>
<td>2.0</td>
<td>1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>1.0</td>
<td>9.9</td>
<td>5.6</td>
<td>3.3</td>
<td>1.6</td>
<td>2.7</td>
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<tr>
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<td>6.6</td>
<td>3.3</td>
<td>2.4</td>
<td>2.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>
quantities of N-acetyl phytosphingosine. The total acetylation (i.e. the sum of N- and O-acetylation, see "Methods") was determined in each of these experiments. The values in Fig. 4 therefore represent, in each case, the sum of N- and O-acetylations. These results show that the total acetylation depended on the relative concentrations of the above two substrates. However at any substrate concentration, the combined acetylation of the two substrates was always smaller than the arithmetic sum of the acetylation of each individual substrate. This again supports the conclusion that both the N- and O-acetylation are catalyzed by the same protein (18).

Reaction Mechanism—As shown in the following paper (17), either the N- or O-acetylation are of the Sequential Bi Bi type.

Identification of Products of Reaction

When using hexadecylamine or the various sphingosine bases, the following compounds might be obtained as products of the acetylation reaction: (a) N-acetyl hexadecylamine; (b) N-acetyl dihydro- or phytosphingosine; (c) partially or fully acetylated LCB's (i.e. mono-, di-, and triacetyl dihydrophosphine or tetraacetyl phytosphingosine). [2-3H3]Acetyl-CoA was incubated with each of the free or N-acetylated bases, the reaction was terminated, and the products were identified with thin layer silica gel chromatography (10, 11) or gas-liquid chromatography of the silylated products (11). Suitable markers were available for the free, N-acetylated, or fully acetylated bases, but not for the intermediate stages where the bases were only partially acetylated. It was of interest that in spite of the many possibilities of the above intermediates only three radioactive spots were obtained on the thin layer plates and only three peaks on gas liquid chromatography column, with dihydrophosphine as substrate (these are herewith designated as "mono-, di-, and triacetyl dihydrophosphine"). With phytosphingosine, four radioactive spots or peaks were obtained (herewith designated as "mono-, di-, tri- and tetracetyl phytosphingosine"). Fig. 5 shows the results of an experiment in which dihydrophosphine, phytosphingosine, and N-acetyl phytosphingosine were incubated with [3H]acetyl-CoA. The positions of the numbers correspond to the relative RF values of the products, and their values represent disintegrations per min. With each of the three substrates, the intermediate products (i.e. the diacetyl dihydrophosphine or di- and triacetyl phytosphingosine) contained the highest radioactivity. In those lanes, where the products were subjected to mild alkaline hydrolysis (i.e. Lanes 8, 9, and 10), the monoacylated product represented (31,000 dpm) was the same without or with mild alkaline hydrolysis. For gas-liquid chromatography, the total product was silylated with the Sigma-AI reagent and chromatographed on 4% SE-30 on Chromosorb, at 230° (11). The results were similar to those obtained by the use of thin layer chromatography.

In a separate experiment, dihydrophosphine was incubated with a mixture of [2-3H]acetate, ATP, CoA, MgCl2, and microsomes which had been stored in the presence of 1 mM DTT. The products were isolated either by thin layer chromatography or by gas-liquid chromatography. The results were similar to those obtained with the same base and [3H]acetyl-CoA.

Distribution of Enzyme—The microsomal LCB transacylase was not found in rat organs (i.e. liver, brain, kidney, or spleen) nor in acetone powder of pigeon livers, although these contained arylamine acetyltransferase. The distribution of the enzyme in 25 strains of the genus Hansenula was presented in a former report (2).

DISCUSSION

This paper describes the properties of a novel enzyme present in the microsomes of the yeast H. ciferri. It exhibited two unique properties: first, it transferred the acetyl group of acetyl coenzyme A to both the amino and hydroxyl groups of the sphingosine bases; none of the other acetyltransferases described to date catalyze both an O- and N-acetyl transfer. Various experiments were performed attempting to distinguish between, and possibly separate the N- and O-acetylating moieties of this enzyme into two individual entities; they were however unsuccessful. It should however be emphasized that the enzyme is not a pure, soluble protein but a microsomal preparation. The possibility that the N- and O-acetylations are catalyzed by two separate membranous proteins can therefore not be excluded. The second unique property of the enzyme was its utilization of the sphingosine bases as acceptors. The enzyme exhibited a rather strict specificity, transferring the acetyl group of acetyl coenzyme A to several sphingosine bases (i.e. sphingosine, dihydrophosphine, and phytosphingosine) and their N-acetyl derivatives as well as to long chain primary amines. p-Nitroaniline, glucosamine, phosphatidylethanolamine, secondary aliphatic amines, primary aliphatic alcohols, or 1-lysolecithin were neither substrates nor inhibitors of the acetylation of the above substrates.
The strict specificity is further emphasized by the finding that among numerous sphingolipids tested as possible substrate, only the above three sphingosine bases and their N-acetyl derivatives were utilized by the enzyme. As the donor, only acetyl coenzyme A was utilized; butyryl-CoA or higher fatty acyl-CoA derivatives were not substrates. This contrast with the acylation of sphingosine in animal systems, where free fatty acids (4, 5) or fatty acyl-CoA derivatives (6, 7) were used to produce N-acyl sphingosine (ceramide). The enzymes which catalyzed these reactions did not acetylate the hydroxyl groups of the bases.

The enzyme could not be detected in animal organs. Furthermore, it was present only in those strains of the yeast which, because of a metabolic aberration of the biosynthetic route, produced excessive quantities of sphingosine bases (2). Its production in these strains is probably a protective mechanism against possible toxic effects of the excessive quantities of the bases produced. Kinetic analyses and conclusions on the mode of interaction of the enzyme with its lipid substrates are presented and discussed in a second, accompanying paper (17).

Acknowledgments—We thank Professor D. Shapiro for gifts of several sphingosine derivatives and Dr. R. M. C. Dawson for the use of the microelectrophoresis apparatus.

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Long Chain Base-Acetyl Coenzyme A Acetyltransferase from the Microsomes of *Hansenula ciferri*: I. ISOLATION AND PROPERTIES
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