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SEROLOGIC AND IMMUNOCHROMATOGRAPHIC DETECTION OF OXYGENATED POLYENOIC ACIDS IN *Euglena gracilis* var. *bacillaris*

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Acetone extracts of *Euglena gracilis* Klebs var. *bacillaris* Cori contain several lipoxygenase and cyclooxygenase products as measured by radioimmunoassay. Three times more of the serologically active products are found in cells grown in the dark than in cells grown in the light. Lack of coincidence of their retention times on high performance liquid chromatography with authentic oxygenation products of arachidonic acid suggest that most of the serologically active compounds are derivitives of polyenoic fatty acids other than arachidonic acid. Several fractions react with anti-12-hydroxyarachidonic acid (an antiserum that recognizes mono- and di-hydroxyarachidonic acids). Three of these fractions have UV absorption spectra characteristic of conjugated trienes.

Euglena gracilis, a unicellular protist, has characteristics of both plants and animals. The cell itself resembles that of a protozoan, but like algae and higher plants, it contains chloroplasts. Like plants and algae, *Euglena* synthesizes linoleic acid and, like animals, it converts the linoleic acid to certain polyenoic acids, including arachidonic acid, which are present in cellular phospholipids (1,2). Since *Euglena* contains the substrate(s) for lipoxygenases and cyclooxygenases, we have examined extracts of *Euglena* cells for the expected products. This paper describes the detection of these compounds in extracts of *Euglena* cells using relatively simple serologic methods.

MATERIALS AND METHODS

Euglena gracilis Klebs var. *bacillaris* Cori were grown, in light or darkness, in Hutner's pH 3.5 synthetic medium in which the carbon sources were glutamic acid and malic acid (3). The cells were collected towards the end of

Abbreviations: 12-HETE, 12-hydroxy-6,8,11,14-eicosatetraenoic acid; iPGE$_2$, immunologically active PGE$_2$; LTB$_4$, leukotriene B$_4$; LTC$_4$, leukotriene C$_4$; LTD$_4$, leukotriene D$_4$; NP-HPLC, normal-phase high performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PGA$_2$, prostaglandin A$_2$; PGE$_2$, prostaglandin E$_2$; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; RIA, radioimmunoassay; RP-HPLC, reverse-phase high performance liquid chromatography; SRS, 6-sulfidopeptide-containing leukotrienes.

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the exponential phase of growth by centrifugation (1500 x g for 10 min). Acetone (250 ml/10^9 cells) was added to the sedimented cells and the mixture was incubated overnight at 25°C in darkness. The suspension was clarified by centrifugation at 1500 x g for 10 min. The acetone extract was dried under negative pressure at 37°C, and the residue was dissolved in 2 ml of 50% (v/v) ethanol. After clarification by centrifugation, the 50% (v/v) ethanol extract was subjected to high performance liquid chromatography.

Materials. Dioleoyl-[1-^{14}C]-phosphatidylcholine (100 mCi/mmol), L-α-1-palmitoyl, 2-arachidonyl, [arachidonyl-^{14}C]-phosphati-dylethanolamine (50 mCi/mmol), and [carboxyl-^{14}C]-tripalmitin (10 mCi/mmol) were purchased from New England Nuclear, Boston, MA. ^{3}H-Labelled standards for the RIA were also purchased from New England Nuclear. Unlabelled phospholipid and triglyceride standards for high performance liquid chromatography were purchased from Sigma Chemical Co., St. Louis, MO.

High-Performance Liquid Chromatography. HPLC was performed by reverse phase (RP)-HPLC on a μ bondapak phenyl column (7.8 x 300 mm, Waters Associates). Waters model 6000A pumps, model 660 solvent programmer and model U6K injector were used for HPLC. Each sample was subjected to chromatography on a linear gradient program from 100% solvent A to 100% solvent B for 100 min at a flow rate of 1 ml/min; 1 ml fractions were collected. Solvent A consisted of 0.01 M phosphate buffer (pH 7.4):methanol (HPLC grade, Fisher):t-amyl-alcohol (Aldrich), (467:30:3, v:v:v). Solvent B was methanol:t-amyl-alcohol (497:3, v:v).

Normal phase HPLC, for the separation of phospholipid classes (4), was performed with isocratic elution. The solvent composition was hexane:2-propanol:25 mM phosphate buffer:ethanol:acetic acid (370:490:62:100:0.6, v:v:v:v:v). The flow rate was 0.5 ml/min at the start and was then changed to 1 ml/min from fraction 60 onwards; fractions were collected each min.

Serologic Analyses. The extracts or HPLC fractions were dried under a nitrogen stream and the residues dissolved in a Tris buffer (0.01 M Tris, 0.14 M NaCl, pH 7.4, containing 0.1% (w/v) gelatin). The serologic specificities of the antisera have been described (5). The 12-HETE antiserum is most effectively inhibited with 12-HETE; however other mono- and di-HETEs also react with anti-12-HETE (6). The anti-LTC_4 immune system reacts with various 6-sulfidopeptide-containing leukotrienes (7).

RESULTS

Acetone extracts of light grown Euglena contain compounds that, after resolution by RP-HPLC, react with antisera directed toward PGE_2, $PGF_{2\alpha}$, 12-HETE and SRS (Fig. 1). The serologically active compounds are heterogeneous with respect to chromatographic properties; only the anti-SRS detect compounds that have retention times approximating those of authentic compounds derived from arachidonic acid. Euglena grown in the dark produce 3-4 times more serologically active compounds than Euglena grown in the light (Table 1). The concentrations of the immunologically active products shown in Table 1 were determined from inhibition curves generated with homologous haptens. However, the acetone extracts of the dark grown Euglena, when subjected to immunochromatography, did produce profiles similar to those shown in Fig. 1. Acetone extracts of bleached mutants of E. gracilis, W_3BUL, a

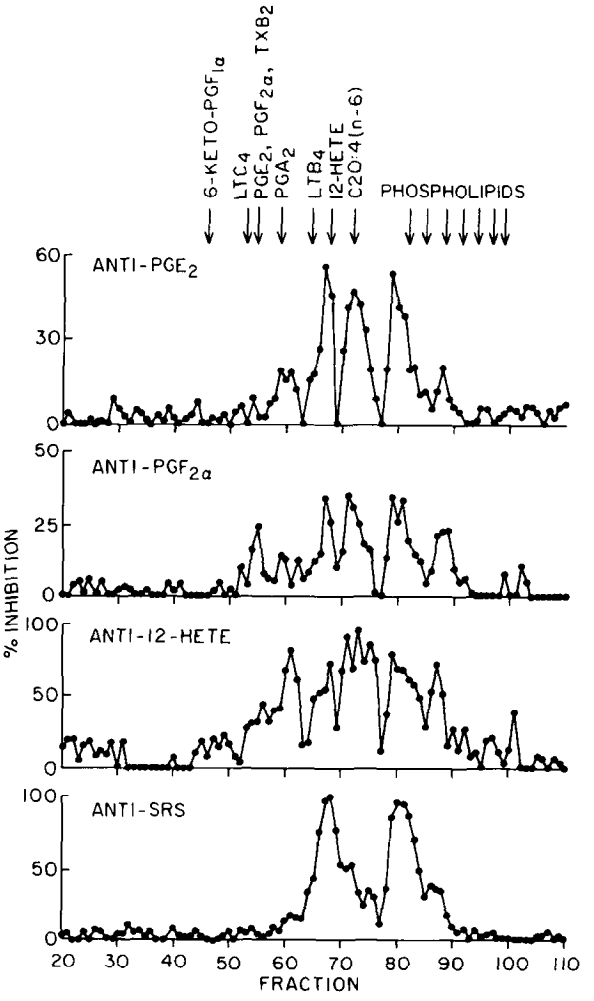

Fig. 1. Immunochromatography (reversed phase HPLC) of an acetone extract of 2.0 x 10^{10} cells of Euglena gracilis grown in the light. Each fraction was analyzed by radioimmunoassay; 200 µl with anti-PGE_2 (top panel); 200 µl with anti-$PGF_{2\alpha}$ (second panel); 100 µl with anti-12-HETE (third panel); and 200 µl with anti-SRS (bottom panel). The data are expressed as % inhibition obtained with the aliquot. The retention times of authentic compounds are designated by arrows.

mutant that produces no detectable amounts of chlorophyll (8) and $W_{10}BSmL$, a mutant that also has no chlorophyll and also lacks proplastids (9), also contain serologically active products equal to that of the dark grown wild type (data not shown). Thus, photooxidation of the polyenoic acids in the presence of chlorophyll does not appear to be responsible for the serologic activity and it is likely that the systems synthesizing the serologically-active compounds are not localized in the plastids.

TABLE 1. IMMUNOLOGICALLY ACTIVE CYCLOOXYGENASE AND LIPOXYGENASE PRODUCTS (AS MEASURED BY RADIOIMMUNOASSAY) OF Euglena gracilis CULTURED IN HUTNER'S pH 3.5 MEDIUM IN THE LIGHT OR DARK

Homologous immune system	Light Grown ng/10^6 cells*	Dark Grown ng/10^6 cells*	dark/light i-activity**
iPGE_2	0.0012	0.0042	3.5
i$PGF_{2\alpha}$	0.0008	0.0026	3.3
i12-HETE	0.525	1.3	2.5
iLTC_4	0.006	0.0167	2.8

* A Coulter electronic cell counter was used for a direct estimation of the population of the culture.

** In each of three experiments, more serologically active products were found in cells grown in the dark than in the light.

In a preparative HPLC of an extract of Euglena that was made to provide material for analyses by UV absorption, at least five broad areas of i12-HETE activity are found (Fig. 2). Some of these serologically active fractions have UV absorption spectra similar to those seen with lipoxygenase products of arachidonic acid (Fig. 3). The compound in Fraction A has two absorption bands in methanol; one at 240 nm, and a second at 267 nm. Compounds in Fractions B, C, and D have UV absorption spectra that are consistent with

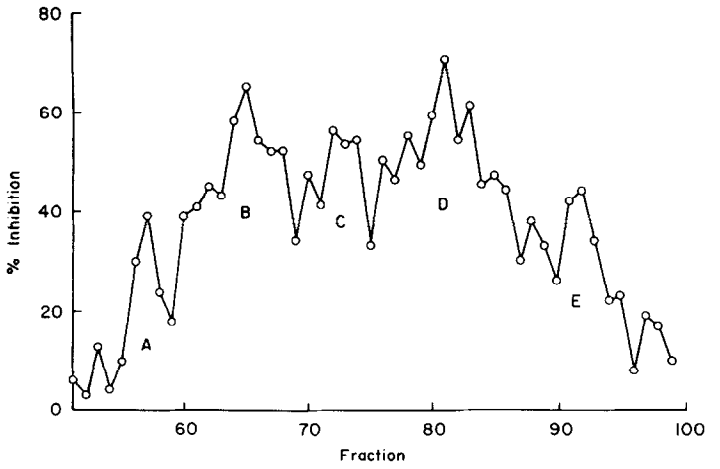

Fig. 2. Immunochromatography for i12-HETE of an acetone extract of Euglena gracilis grown in the light. For RP-HPLC a preparative μ Bondapak phenyl column was used. For RIA with anti-12-HETE, 20 μl of each fraction was used.

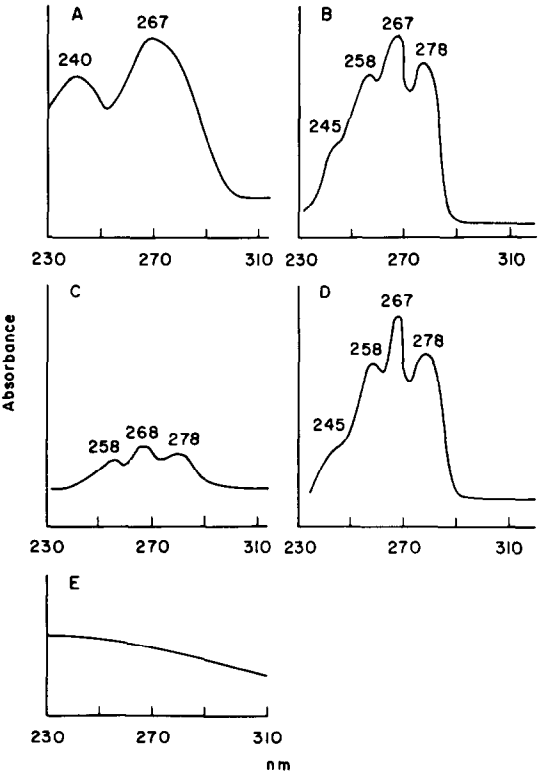

Fig. 3. Ultraviolet absorption spectra of serologically active fractions (A, B, C, D, E of Fig. 2). UV absorption spectra were obtained using a Perkin-Elmer 559 UV-VIS spectrophotometer.

those of conjugated trienes. The compound in Fraction E, although it reacts with the anti-12-HETE, does not absorb UV light, at least not at the concentrations tested. The compound in Fraction B has a retention time of 66 min, similar to that of authentic LTB_4. The serologically active compounds in Fraction D (Fig. 2, 3) also have UV absorption spectra similar to that of a conjugated triene; but their retention times (78 to 85 min) are longer than that of authentic LTB_4. Their retention times are similar to that of authentic phospholipids. The fractions comprising fraction D (Fig. 2), therefore, were pooled, dried under a stream of nitrogen and subjected to NP-HPLC for separation of phospholipids. The serologic activity of the rechromatographed products as well as the retention times of authentic phospholipids are shown in Fig. 4. The serologic activity elutes in a single sharp peak with a retention time of 11 min, close to that of authentic phosphatidylethanolamine

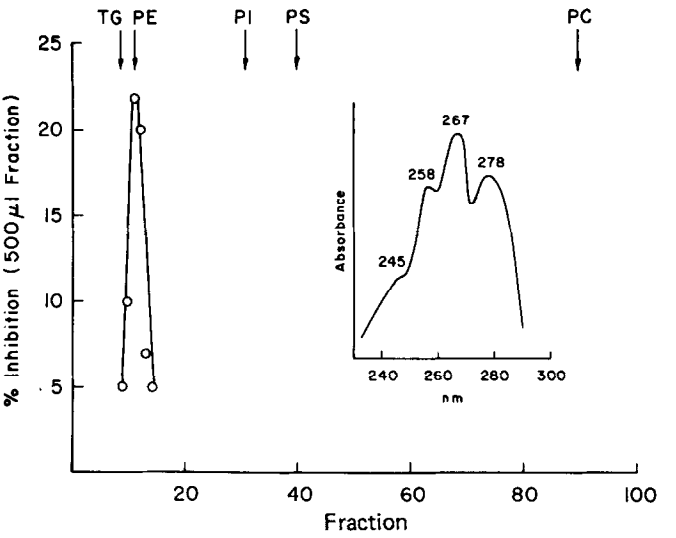

Fig. 4. Immunochromatography (NP-HPLC-RIA) of Fraction D (Fig. 2). A µ-Porasil column was used. Fraction D was chromatographed for 120 min. Retention times of authentic standards are designated by the arrows. 500 µl of each fraction was assayed for serologic activity with anti-12-HETE. The inset contains the UV absorption spectrum of the serologically active product.

(and cholesterol esters, J. Lowenstein, Personal Communication). Authentic phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylcholine (PC) elute much later, whereas triglycerides (TG) elute at 8 min. The UV absorption spectrum of the serologically active compound is shown in the inset (Fig. 4). It has a λ_{max} at 267 nm with shoulders at 245 nm, 258 nm and 278 nm.

DISCUSSION

Arachidonic acid, the polyunsaturated fatty acid present in the highest concentrations in the membranes of eukaryotic cells, is a substrate for lipoxygenases and cyclooxygenases but it is not the only polyenoic fatty acid that can serve as substrate (10,11). Euglena gracilis has the genetic capacity to synthesize a wide variety of long chain polyunsaturated fatty acids, whether grown in the light or in the dark (1, 13-17). Cells grown in the dark, however, contain higher levels of potential substrates for cyclooxygenase and lipoxygenase activities than cells grown in the light; and there is an increase in both cyclooxygenase and lipoxygenase products extracted from cells grown in the dark suggesting that the prostaglandin-, HETE- and

SRS-like substances found in Euglena are products of its animal-like fatty acid metabolism. Growth in darkness which reduces the chloroplasts to proplastids increases the amount of mitochondrial material compared with light grown cells which contain chloroplasts (22). Antibody specificity with respect to arachidonic acid metabolism is directed toward functions distal to the common carboxyl function used to synthesize the immunogen (18,19). The fatty acid chain proximate to that carboxyl function adds little to the considerable binding energy of the eicosanoid-antibody complex. The immunoreactive compounds in extracts of Euglena probably have some or all of the structures recognized by the appropriate antibodies although they probably are derivatives of arachidonic acid metabolites such as have been identified in Plexaura homomalla (20,21) or products synthesized from different polyunsaturated fatty acid substrates. Thus, it is not surprising that, when using immunochromatographic analysis, several peaks of PGE_2, $PGF_{2\alpha}$, 12-HETE, and SRS serologic activity are found in cells of Euglena gracilis var. bacillaris (Fig. 1). Of course, absolute identification of these serologically active compounds must await GC-MS analysis.

The compounds measured by the antiserum to the arachidonic acid lipoxygenase product, anti-12-HETE, were examined in greater detail, especially two fractions. One has a retention time similar, but not identical, to that of an arachidonic acid metabolite, LTB_4. The serologically active compounds in Fraction D migrate with authentic phosphatidylethanolamine (and cholesterol esters, J. Lowenstein, Personal Communication). In view of the fact that E. gracilis contains several sterols (23), it is possible that the conjugated triene-containing compound in Fraction D is associated with a sterol. Alternatively, the conjugated triene-containing compound in Fraction D may be present in phosphatidylethanolamine, most likely in the 2-acyl position. When the compound in Fraction D is used as a substrate for phospholipase A_2 or phospholipase C, no change is observed in the chromatographic properties of the metabolite, or in its UV absorption spectrum suggesting that it is not present in the 2-acyl position of phosphatidylethanolamine. If it is ester-

ified to a sterol, it is unlikely that the sterol is ergosterol (24,25), judging from its UV absorption spectrum, since this sterol has a distinctive absorption spectrum and its extinction coefficient approximates that of conjugated trienes in the 200-300 nm region of the spectrum.

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