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REVIEW

Fluorescence-Based Assays of Lipases, Phospholipases, and Other Lipolytic Enzymes[1]

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Lipolytic enzymes have traditionally been assayed by radiometric and titrimetric methods (for a review of phospholipase assays see Reynolds *et al.* (1)). Radiometric assays are the most sensitive, but they require the use of expensive radiolabeled substrates, are discontinuous, and require separation of the radioactive substrate from the labeled products. Such separations are often tedious, and the safe use of radioactive materials is of increasing concern. Titrimetric assays are continuous and quite straightforward; natural or synthetic substrates can be used, but they suffer from low sensitivity and are subject to conditions that could alter the amount of free hydrogen ion released. These considerations have given rise to the development of fluorescence-based assays. Fluorescence methods have sensitivities that approach those of radiometric ones; however, they require synthetic fluorescent-labeled substrates or probes. Traditional fluorometric assays are discontinuous and, like radiometric methods, require the separation of substrate from products. More recently, continuous fluorescence-based assays have been developed which rely on changes in the fluorescence properties of the substrate upon hydrolysis. In the future, chemiluminescent assays hold the promise of even higher sensitivity, but require the clever design of synthetic substrates.

In this review, fluorescence-based assays of lipolytic enzymes are classified according to two basic types: discontinuous and continuous. Within each category, assays are presented according to the type of fluorophore used, rather than the particular enzyme; each method may be applicable to more than one enzyme. Table 1 classifies assays according to the enzyme and lists the fluorophore and references to them. Structures of some representative fluorescent-labeled lipids, abbreviated in bold type in the text, are shown in Scheme 1.

GENERAL CONSIDERATIONS

For the assay of any enzyme, one has to consider sensitivity, the availability of substrates, and the ease of the procedure. Since lipolytic enzymes act preferentially at lipid interfaces, one also has to consider the physical state of the substrate. In fluorescence-based assays the perturbing effects of the fluorophore are an additional consideration. This could involve the intrinsic substrate specificity of the enzyme or the interfacial properties of the substrate. Continuous fluorescence assays usually depend on a change in the fluorescence properties of the probe as the substrate is converted to product. This often involves change from a nonpolar environment of the probe in the substrate to a polar environment of the probe in the product. Dansyl[2] and NBD groups, which are particularly sensitive to polarity, are often used in these assays. Detergents, which are often necessary to create a substrate interface suitable for enzyme activity, may have significant effects on the fluorescent properties of fluorophores in substrates and products. Fluorescent-based assays, therefore, are highly intolerant of

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[2] Abbreviations used: BODIPY, 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*S*-indacene; dansyl, 5-dimethylamino-naphthalene-1-sulfonyl; DPybPC, 1,2-bis-pyrenebutanoyl-*sn*-glycero-3-phosphocholine; LRO-GC, lissamine rhodamine sulfonylamido-sphingosyl-β-glucose; LUMI-PI, (±)3-(4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl-*myo*-inositol-1-phosphate; NBD, 7-nitrobenzo-2-oxa-1,3-diazol-4-yl; NVPC, 1-*O*-[12-(2-naphthyl)-dodec-11-enyl]-2-*O*-decanoyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI-PLC, phosphatidylinositol-specific phospholipase C; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C; pyrene-PI, 4-pyrenebutylphosphoryl-1-*myo*-inositol; UMB, umbelliferone; VLDL, very-low-density lipoprotein.

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TABLE 1

Fluorescence-Based Assays of Lipolytic Enzymes

Enzyme[a]	Method	Fluorophore	References
Cholesterol esterase	Discontinuous	Pyrene	20
Glucosyl-ceramide glucosidase	Discontinuous	NBD	16–18
		Lissamine rhodamine sulfonyl	10
LCAT	Continuous	Pyrene	49
Lipase	Continuous	Coumarin	4, 32
		Dansyl	35–39
		NBD	42, 43
		Pyrene	52–55
	Discontinuous	Pyrene	20–24
PAF metab. enzymes	Discontinuous	Dansyl	7
		7-Methoxycoumarin	11
PLA_1/PLA_2	Continuous	BODIPY	31
		Dansyl	38, 40, 41
		NBD	42–45
		Pyrene	3, 47, 48, 50
	Discontinuous	9-Anthracenylmethyl	6
		Dansyl	7–9
		NBD	13–15
		Naphthylvinyl	8, 9, 12
		Pyrene	19
PLC	Continuous	Dansyl	39
		Pyrene	51
PI-PLC	Continous	Naphthyl	46
	Discontinuous	Dioxetane	5
		Pyrene	29, 30
Sphingomyelinase	Continous	Coumarin	33, 34
	Discontinuous	Pyrene	21, 25–28

[a] LCAT, lecithin:cholesterol acyltransferase; PAF, platelet-activating factor; PLA_1/PLA_2, phospholipases A_1 and A_2; PLC, phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C.

changes in detergent or other factors that may affect the substrate interface. There are, therefore, severe limitations in the use of these methods in the study of interfacial effects on enzyme activity.

Pyrene, as a fluorophore in enzyme assays, is attractive not only because of its high emission intensity, but also because of its excimer emission; as a monomer, it emits at 382 and 400 nm, but as an excited dimer its emission shifts to 480 nm. The synthesis of 1,2-bis[10-(1-pyreno)decanoyl]-*sn*-glycero-3-phosphocholine and the characterization of its fluorescent properties by Sunamoto *et al.* (2) suggested its use in a continuous fluorometric assay of phospholipase A_2 (PLA_2) (3).

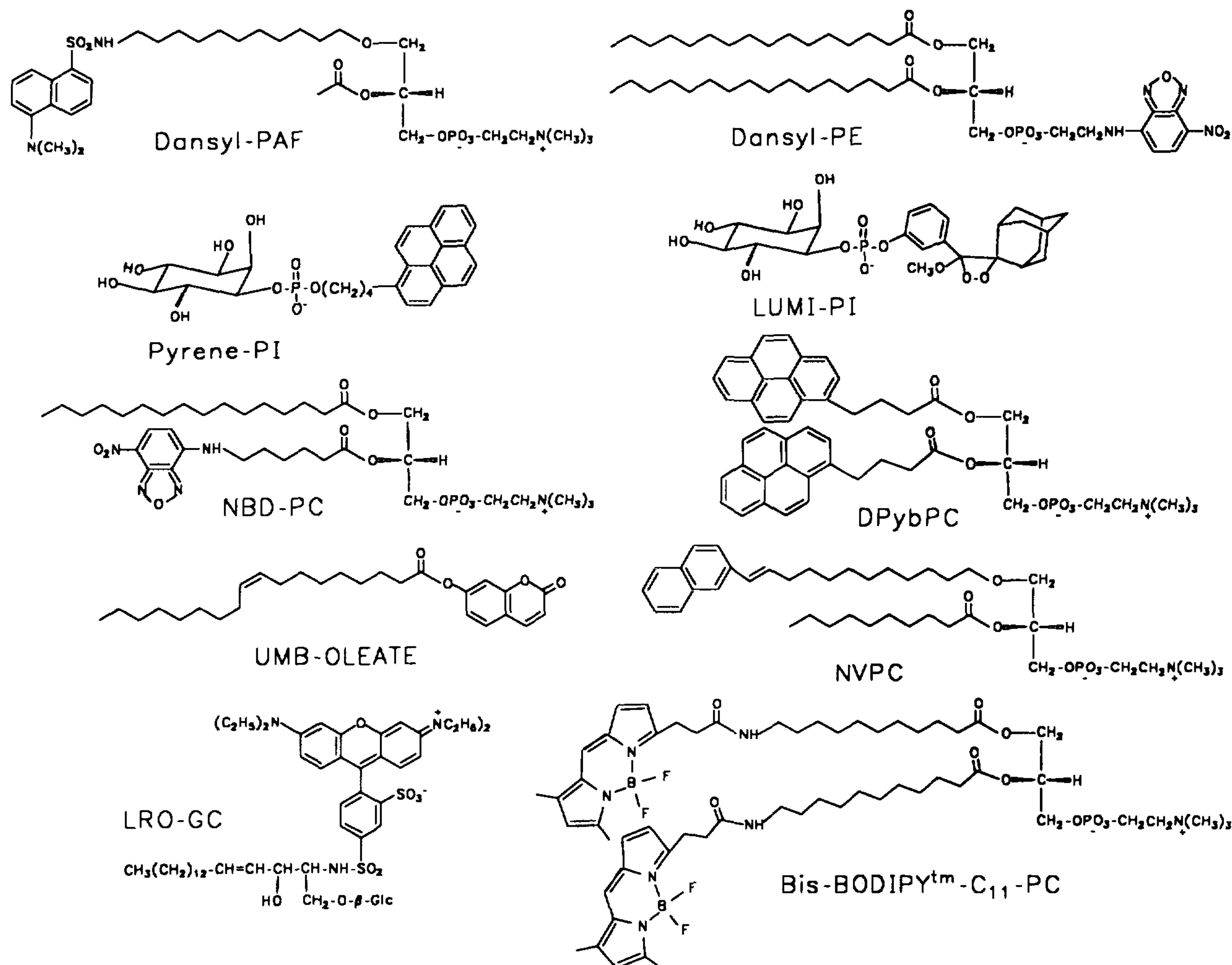
The use of a substrate that is not fluorescent, but becomes fluorescent upon hydrolysis, is particularly attractive due to the high degree of sensitivity and the ease of a continuous assay. A coumarin derivative, 4-methylumbelliferone, was first used in a lipase assay (4). When esterified to a fatty acid, the coumarin derivative is not fluorescent; after hydrolysis, the free hydroxycoumarin is highly fluorescent. The presence of a bulky coumarin ring, however, may render the substrates less active. These compounds can also be hydrolyzed by nonspecific carboxylesterases.

Chemiluminescent assays are even more sensitive than fluorescent assays. A water-soluble, chemiluminescent substrate (**LUMI-PI**) for the assay of phosphatidylinositol-specific phospholipase C (PI-PLC) was recently synthesized (5). The product of hydrolysis, a labile anion, decays with the emission of light. Although these substrates are subject to the same limitations as discussed above for coumarin derivatives, their extremely high sensitivity in assays makes them very attractive for such purposes as screening cultures of enzyme-producing bacteria and visualizing enzyme activity in a biological membrane.

DISCONTINUOUS ASSAYS

Anthracenylmethyl-Labeled Lipids

Tojo *et al.* (6) recently described a HPLC-based assay of PLA_2 in which fatty acids released by the enzyme were extracted and derivatized with 9-anthryl-diazomethane prior to HPLC separation and uv detection. Margaric acid was included as an internal standard. This is a sensitive assay that uses natural phospholipids as substrates, and the individual fatty acids liberated can be identified by HPLC. It can also be adapted to the



SCHEME 1. Structures of some representative fluorescent-labeled lipids (bold type in text). PAF, platelet-activating factor; PE, phosphatidylethanolamine; PI, phosphoinositide; PC, phosphatidylcholine; DPybPC, bis-pyrenebutanoyl-phosphatidylcholine; UMB, umbelliferone; NVPC, naphthylvinyl-phosphatidylcholine; LRO-GC, lissamine rhodamine sulfonylamido-sphingosyl-β-glucose.

assay of other lipolytic enzymes such as lipases and lysophospholipases.

Dansyl-Labeled Lipids

Schindler *et al.* (7) designed dansyl-labeled platelet-activating factor (**PAF**) analogs for the assay of PLA_2 and enzymes of the PAF cycle. The substrate, 1-(*N*-dansyl-11-aminoundecyl)-2-acetyl-PC (**Dansyl-PAF**), is a PAF analog with an ether linkage at the *sn*-1 position that is not enzymatically hydrolyzed. The dansyl group remains attached to the glycerol backbone, so that metabolic products of the substrate can be identified and measured. They used this substrate, and its lyso analog, to follow the activities of lyso-PAF acyltransferase, lyso-PAF acetyltransferase, and PAF acetylhydrolase in polymorphonuclear leukocytes; the products were separated by TLC and measured by fluorescence scanning. Hendrickson *et al.* (8,9) used the *sn*-2-decanoyl analog as a substrate for the assay of PLA_2. The substrate and product were separated by TLC and measured by fluorescence scanning. This assay is simple and requires no standards since the product/(substrate + product) ratio can be directly determined by fluorescence scanning.

Dioxetane-Labeled Lipid

Ryan *et al.* (5) recently synthesized a water-soluble, chemiluminescent substrate, **LUMI-PI,** for the assay of PI-PLC. The diacylglycerol moiety of natural phosphatidylinositol was replaced by a dioxetane-containing moiety. PI-PLC-catalyzed hydrolysis of this substrate results in the formation of a chemiluminescent precursor, which decays with the emission of light. The assay procedure uses 96-well microtiter plates with detection

of the chemiluminescence on autoradiography film. The detection limit was about 10 pg of pure PI-PLC. Assays can also be carried out using a luminometer. Activity was readily detected from small volumes (<1 μl) of liquid cultures of PI-PLC-producing bacterial strains; thus, this assay can be used to screen PI-PLC-producing bacterial strains.

Lissamine Rhodamine Sulfonyl-Labeled Lipids

Agmon *et al.* (10) synthesized a novel lissamine rhodamine sulfonylamido-sphingosyl-β-glucose (**LRO-GC**) and -sphingosyl-β-glucosyl-β-galactosyl-α-galactose as substrates for the lysosomal acid β-galactosidase. The product, LRO-ceramide, which cannot be further hydrolyzed, was separated from the substrate by TLC and quantitated by fluorescence emission. Both glycolipids penetrated the membrane of intact fibroblasts and were hydrolyzed in lysosomes of intact cells. These substrates were useful in subtyping Gaucher disease variants.

7-Methoxycoumarin-Labeled Lipids

Mita *et al.* (11) described a HPLC method to quantitate platelet-activating factors. Samples, with the addition of a 2-propionyl analog as an internal standard, were hydrolyzed with PLC, and derivatized with 7-methoxycoumarin-3-carbonyl chloride or 7-methoxycoumarin-4-acetic acid to form 7-methoxycoumarin ester derivatives. The products were separated by HPLC and detected by fluorescence. The lower limit of detection was about 100 pg. The method is applicable to the assay of PAF acetylhydrolase activity in polymorphonuclear leukocytes.

Naphthylvinyl-Labeled Lipids

Hendrickson *et al.* (8,9,12) synthesized a naphthylvinyl-labeled glycerol ether analog of phosphatidylcholine, **NVPC,** as a substrate for the assay of PLA_2. Like the dansyl-labeled PAF analog described above, this lipid has an ether linkage at the *sn*-1 position and is not hydrolyzed by PLA_1. The substrate and product were separated by HPLC and their ratio was determined by fluorescence detection. Activities as low as 1 pmol/min in an assay volume of 50 μl could readily be detected. The naphthylvinyl probe is nonpolar and nonperturbing; the PAF analog was almost as active as natural PAF for platelet aggregation.

NBD-Labeled Lipids

Dagan and Yedgar (13) developed an assay for PLA_2 using 1-acyl-2-(NBD-aminohexanoyl)-phosphatidylcholine (**NBD-PC**) and a simple solvent extraction to separate free NBD-hexanoic acid from the substrate. The amount of fatty acid was determined by its fluorescence emission. NBD-hexanoic acid was preferred over other fluorescent products, since it was not incorporated into other lipids and its fluorescence was fully recovered. Slightly modified, the method was used to measure PLA_2 activity in Epstein–Barr virus-transformed lymphoblast cells (14). Saris and Somerharju (15) used the NBD label in a different way to assay for PLA_2 acting on biomembrane phospholipids. After incubation of biomembranes with PLA_2, the lipids were extracted, and PE and lyso-PE were derivatized with NBD-F (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole). This is a fairly simple method, and has the advantage that only endogenous membrane phospholipid is used as substrate; labeled, exogenous lipid analogs may not have access to all cellular membranes.

A simple, sensitive assay of glucosylceramide β-glucosidase used a fluorescent NBD-analog of glucosylceramide (an amide of glucosylsphingosine and NBD-decanoic acid) as a substrate (16,17). The fluorescent product (NBD-ceramide) was separated from the substrate by extraction. The method was modified (18) by adding a free-radical trapping agent, SlowFade (Molecular Probes, Inc., Eugene, OR), to prevent bleaching of the NBD compounds, and standards were prepared by complete enzymatic hydrolysis of measured amounts of NBD-substrate.

Pyrene-Labeled Lipids

Thuren *et al.* (19) used the fluorescent phospholipid analog 1-octacosanyl-2-pyrenehexanoyl-*sn*-glycero-3-phosphatidyl monomethyl ester as a substrate for the fluorometric assay of PLA_2 in serum to diagnose acute pancreatitis. The use of a 1-alkyl ether group excluded any hydrolysis by PLA_1; the methylphosphate head group was selected since it gave the highest activity with pancreatic PLA_2. The fluorescent product, pyrenehexanoic acid, was separated from the unreacted substrate by liquid–liquid phase partition. The amount of product was determined by its fluorescence emission compared with standards.

Negre *et al.* (20) used 1,2-dioleoyl-3-pyrenedecanoylglycerol for a fluorometric assay of lysosomal acid lipase in the diagnosis of Wolman and cholesteryl ester storage diseases. The pyrene-labeled fatty acid product was separated by partition between chloroform–methanol–water, and the fluorescence emission was compared with standards. They also used pyrenedecanoyl cholesterol as a substrate for the assay of cholesterol esterase, and reported significant enhancement of fluorescence of these pyrene-labeled lipids by polar media, detergents, and phospholipids (21). In a later study, Negre *et al.* (22) used pyrene decanoic acid to label normal and acid-lipase deficient cultured fibroblasts. The fluorescence intensity emitted by lipidotic cells exceeded that of the normal cells due to increased fluorescence from

the lysosome-mitochondrial fraction. They also used pyrene-labeled triacylglycerols to characterize lipases from human stomach and gastric juice (23), pancreatic carboxylic ester hydrolase, and bile salt-stimulated lipase (24).

N-Pyrenedecanoyl-sphingomyelin was used in a fluorescent assay of sphingomyelinase activity in Epstein–Barr virus-transformed lymphoid cell lines established from patients with Niemann–Pick disease (25). After the uptake of this lipid by fibroblasts from normal and Niemann–Pick disease individuals, fluorescent ceramide was seen only in normal cells (26). *N*-12-(1-Pyrenesulfonylamido)dodecanoyl-sphingomyelin was also used as a substrate for determining sphingomyelinase activity (27). These two fluorescent lipids and pyrene-propenoyl-sphingomyelin were used to study the mode of uptake and degradation by skin fibroblasts from normal individuals and patients (28).

Hendrickson *et al.* (29,30) synthesized 4-pyrenebutyl-phosphoryl-1-*myo*-inositol (**Pyrene-PI**) as a substrate for the assay of PI-PLC from *Bacillus cereus*. The fluorescent substrate and product were separated and quantified by HPLC with fluorescence detection. The maximal activity with micelles of pyrene-PI was estimated to be 68 μmol min^{-1} mg^{-1} or about 4% of the activity with natural substrate. Rates as low as 10 pmol/min (about 0.2 ng of enzyme) could readily be detected.

CONTINUOUS ASSAYS

BODIPY-Labeled Lipids

Bis-BODIPY-C_{11}-PC (Molecular Probes, Inc.) contains BODIPY (4,4-difluoro-4-bora-3*a*,4*a*-diaza-*S*-indacene)-labeled undecanoic acid esterified to the *sn*-1 and *sn*-2 positions of phosphatidylcholine. PLA_1- or PLA_2-catalyzed hydrolysis of this lipid relieves the self-quenching of the BODIPY fluorophores, resulting in increased fluorescence emission. Meshulam *et al.* (31) used this lipid to continuously monitor PLA activity in intact polymorphonuclear leukocytes by flow cytometry. Using indo-1 simultaneously to measure Ca^{2+} levels, a rise in intracellular Ca^{2+} was shown to precede PLA activation in immune complex-stimulated cells. The BODIPY probe has a high extinction coefficient and quantum yield, and is spectrally compatible with argon-laser excitation sources. Other BODIPY-labeled lipids, available from the same source, should prove useful in fluorescence-based assays.

Coumarin-Labeled Lipids

Jacks and Kircher (4) used fatty acyl esters of 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) in a continuous fluorometric assay of lipase. De Laborde de Monpezat *et al.* (32) later found umbelliferone to be more sensitive and stable than 4-methylumbelliferone, and used fatty acyl esters of the former to measure lipase activity. Umbelliferyl oleate (**UMB-oleate**) was a very good substrate for *Rhizopus delemar* and *Candida cylindracea* lipases.

Jones *et al.* (33) reported on the hydrolysis of bis(4-methylumbelliferyl)phosphate by purified placental sphingomyelinase. Hydrolysis was greatly stimulated by addition of Triton X-100, although the compound was not incorporated into the Triton micelles. They observed nonhyperbolic kinetic patterns and, because of this, advised against the use of this substrate in sphingomyelinase assays. When 4-methylumbelliferyl-phosphocholine was used as a fluorogenic substrate for purified placental sphingomyelinase, hydrolysis was independent of any added detergent (34).

Dansyl-Labeled Lipids

Johnson *et al.* (35) used *N*-dansyl phosphatidylethanolamine (**Dansyl-PE**) as a probe to monitor lipolysis in very-low-density lipoproteins (VLDL). Addition of lipoprotein lipase to dansyl-PE-VLDL, in the presence of albumin, resulted in a more than threefold increase in fluorescence emission and a blue shift of 20 nm in the wavelength of maximum emission. These changes followed the same time course as the hydrolysis of triglycerides and the release of fatty acids. No fluorescent changes occurred when lipoprotein lipase was added to either low- or high-density lipoproteins labeled with dansyl-PE. Fluorescent changes in dansyl-PE were shown to be due to its hydrolysis, catalyzed by lipoprotein lipase, and the subsequent binding of lyso-dansyl-PE to albumin (36). The catabolism of human VLDL by bovine milk lipoprotein lipase has been monitored by this method (37).

Wilton (38) described a continuous fluorescence displacement assay of PLA_2 and other lipases that release long-chain fatty acids. This involves the displacement of dansyl-amino-undecanoic acid from rat liver fatty acid-binding protein by the long-chain fatty acid products of hydrolysis. The initial rate of decrease in fluorescence intensity was linearly proportional to enzyme activity; the assay could detect rates as low as 10 nmol min^{-1} ml^{-1}, and could be used with any substrate and enzyme that catalyze the release of a long-chain fatty acid. Fatty acid-binding protein cannot be replaced by serum albumin, and higher concentrations of substrate require correspondingly higher concentrations of both the binding protein and the dansyl-amino-undecanoic acid. Detergents will probably reduce the effectiveness of fatty acid binding and can not be tolerated in this assay. As little as 10 pg/ml of pure pancreatic lipase was detected with low concentrations of substrate (50 μg/ml of olive oil), and as little as 2 ng/ml of pure PLC by coupling the enzyme to excess lipase (39). The latter assay would be compromised by the presence, in the

substrate, of small amounts of other lipids hydrolyzed by lipase. Kinkaid and Wilton (40) used this system to compare the catalytic properties of PLA_2 from pancreas and venom with substrates containing a variety of fatty acids and different headgroups. With limiting amounts of substrate (0.1–1 μM), stoichiometric conversion of substrate into products was observed.

Recently, Kinkaid and Wilton (41) described a modification of their fluorescence displacement assay for PLA_2 using albumin and medium-chain phospholipid substrates. The procedure involves the displacement of dansyl-amino-undecanoic acid from albumin by decanoic acid released upon hydrolysis of didecanoyl-PC. As little as 1 ng of pure cobra venom PLA_2 could be detected. The release of unsaturated fatty acids, e.g., oleic and arachidonic acid, could also be detected by fluorescence displacement, but only after a lag period. The albumin-linked procedure is better suited for the routine assay of small amounts of PLA_2; the fatty acid-binding protein method is better suited for kinetic studies.

NBD-Labeled Lipids

Wittenauer *et al.* (42) used 1-acyl-2-(NBD-hexanoyl)-PC (**NBD-PC**) as a substrate for the continuous assay of PLA_2 and lipoprotein lipase; the latter enzyme also exhibits PLA_1 activity. Hydrolysis, catalyzed by either enzyme, resulted in an increase in emission, with no shift in the wavelength of maximum emission. A discontinuous assay under the same conditions, with separation of the products by TLC and quantitation by fluorescence, showed a decrease in labeled substrate, an increase in NBD-hexanoic acid for PLA_2, and an increase in lyso-NBD-PC for lipoprotein lipase. PLA_2 activity also resulted in a small increase in fluorescent lyso-NBD-PC, whereas lipoprotein lipase activity also resulted in an increase in NBD-hexanoic acid. The latter results are unexpected and raise questions about the validity of this assay. Commercial 1-acyl-2-(NBD-hexanoyl)-PC may be contaminated with an isomer in which the NBD-hexanoyl group is in the *sn*-1 position, since it is made from lyso-egg-PC, which is known to isomerize by acyl migration. This would explain the appearance of fluorescent lyso-NBD-PC after PLA_2 activity, and NBD-hexanoic acid after lipoprotein lipase activity.

Moreau (43) evaluated NBD-phospholipids as substrates for the assay of phospholipases and lipase. With PLA_2, the rates of hydrolysis of NBD-phospholipids were several hundred-fold lower than the rates of hydrolysis of natural phospholipids. With lipases, the rates of hydrolysis of NBD-PC were extremely low, so that a small contamination with phospholipases would be misleading. He also showed that detergents and serum albumin interfered with the assays in a concentration-dependent manner. Meyuhas *et al.* (44) reported severe limitations on the use of NBD-PC for PLA_2 assays. The low emission intensity of aggregated NBD-PC is due to self-quenching, which is maximal in the absence of other lipids. The emission intensity increase after hydrolysis is due to diffusion of NBD-hexanoic acid to the aqueous phase where it exists as monomers. In the presence of additional lipids (mixed micelles, membrane vesicles, emulsified lipid or lipoproteins), the probe may be only partially quenched, and the fluorescent product may not completely partition into the aqueous phase. Since complex changes in fluorescence emission could result (sometimes a decrease), the continuous assay of PLA_2 activity with NBD-PC is ambiguous.

Monti *et al.* (45) reported real-time fluorescence polarization measurements to follow the PLA_2-catalyzed hydrolysis of 1-acyl-2-(NBD-dodecanoyl)-PC. The procedure required modification of a SLM 4800 spectrofluorometer to allow continuous measurement of fluorescence polarization and maintenance of a low substrate concentration ($<3\ \mu M$) to avoid aggregation. The method is subject to the same limitations as reported by Meyuhas *et al.* (44) (see above), and requires sophisticated instrumentation. Nevertheless, with the advent of newer fluorescence instrumentation capable of polarization measurements, this is an interesting approach, and might be applicable to other, less-perturbing fluorescent probes.

Naphthyl-Labeled Lipids

Shashidhar *et al.* (46) used 2-naphthyl *myo*-inositol-1-phosphate as a substrate for the continuous assay of PI-PLC. The specific activity of PI-PLC with this substrate was only about 0.003% that with the natural substrate. Because of its low activity, and its limitations as a water-soluble, monomeric substrate, its use in the assay of PI-PLC is restricted.

Pyrene-Labeled Lipids

Hendrickson and Rauk (3) first described a continuous assay of PLA_2 using 1,2-bis-pyrenebutanoyl-PC (**DPybPC**) as the substrate. The substrate gives only excimer emission at 480 nm, while the lyso-PC product gives only monomer emission at 382 and 400 nm; hydrolysis was followed by the increase in monomer emission at 382 nm. The substrate has a critical micelle concentration of 7.3 μM, so activity can be measured under monomolecular or micellar conditions. Thuren *et al.* (47) observed that DPybPC could not be used for the assay of PLA_2 in the presence of serum albumin, because in its presence, the unhydrolyzed substrate gives monomer emission indistinguishable from that of the product. This was also the case with 1-triacontanyl-2-pyrenehexanoyl-PC. However, Radvanyi *et al.* (48) found that PLA_2 activity can be continuously monitored in the presence of serum albumin, using PC labeled in

the *sn*-2 position with pyrenedecanoic acid. The pyrenedecanoic acid product is tightly bound to serum albumin so that only monomer emission is observed. This assay can detect picogram amounts of PLA_2 in crude extracts. Bonelli and Jonas (49) used DPybPC as a substrate for the assay of lecithin:cholesterol acyltransferase; the esterase activity was determined by following the increase in monomer emission as submicellar concentrations of substrate were hydrolyzed.

Thuren *et al.* (50) used 1-palmitoyl-2-pyrenehexanoyl-phosphatidyl-*N*-(trinitrophenyl)-ethanolamine in an assay of PLA_2. The pyrene fluorescence is intramolecularly quenched by the trinitrophenyl group (suggesting that the pyrene ring is situated close to the interface). Upon hydrolysis, the monomer emission increased as pyrenehexanoic acid was released into the aqueous phase and quenching was relieved. They later used this same substrate for a continuous fluorometric assay of PLC (51).

Negre *et al.* (52) used pyrenemethyl laurate as a fluorescent substrate for the continuous assay of lipase. Dispersions of the compound in dimethyl sulfoxide or taurocholate produced only excimer emission, while the product of hydrolysis, pyrenemethanol, yielded monomer emission. The method has been applied to gastric lipase, cellular lipases of hemopoietic cells, and the bacterial lipase of *Rhizopus arrhizus,* and to diagnose acid lipase deficiency in Wolman's and cholesterol ester storage diseases (53,54). Liodakis *et al.* (55) used 1-pyrenebutanoyl-2,3-bis-palmitoyl-glycerol as a substrate for the continuous assay of lipase. They monitored an increase in monomer emission as the product, pyrenebutanoic acid, diffused into the aqueous phase.

SUMMARY

In choosing an assay, one needs to consider the following questions: What level of sensitivity is required? Must the assay be continuous? Is the substrate readily available; can it be purchased or must it be custom synthesized? How specific is the substrate? How convenient is the method? How compatible is it with monomolecular, micellar, or vesicular substrates? How tolerant is it of added detergents and proteins that may be present? What is the cost of substrates, fluorescent probes, and instrumentation?

Of the many methods described in this review, discontinuous assays using natural substrates and derivatization of the products with fluorescent probes are probably the most reliable and most tolerant of reaction conditions. A drawback is the involvement of tedious and time-consuming steps which limit the number of trials that can be performed. Continuous assays, in which changes in fluorescent properties of the probe are monitored, are most convenient for kinetic studies, although they are also most sensitive to reaction conditions and intolerant of added detergents and proteins. One has to carefully consider all of these issues and choose a method best suited to the enzyme, the particular information one wants to obtain, and the availability of substrates, probes, and instrumentation. Hopefully, increased commercial availability of fluorescent substrates and probes will make these choices easier. Nevertheless, the search goes on for better, more sensitive and convenient fluorescent assays.

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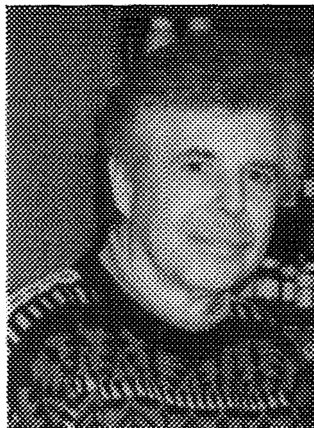

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