

An Evaluation of NBD-Phospholipids as Substrates for the Measurement of Phospholipase and Lipase Activities

Because most of the existing assays of phospholipase activity are quite laborious, the use of 1-acyl-2-[6-(7-nitro-1,3-benzoxadiazol-4-yl)amino]caproyl labeled phospholipids (NBD phospholipids) was investigated to determine whether they could be used as substrates in the routine assay of various phospholipases and lipases. NBD-labeled analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidic acid were evaluated. There was about a 50-fold increase in fluorescence upon hydrolysis of the NBD hexanoic acid from the NBD phospholipid, confirming an earlier report. This change in fluorescence was constant over the normal physiological pH range (pH 5-9). Detergents and bovine serum albumin interfered with the assay in a concentration dependent manner. An increase in fluorescence and a concomitant increase in NBD hexanoic acid was detected with the two phospholipase A₂ enzymes. Although a change in fluorescence was detected with a phospholipase C, careful evaluation revealed that the rate of increase in fluorescence was not proportional to the rate of production of diacylglycerol product. Neither of the two phospholipase D enzymes which were tested were able to cause an increase in fluorescence when incubated with NBD phospholipids. A small increase in fluorescence was detected with each of the four lipases. Of the five NBD lipids tested, the highest rates of hydrolysis were consistently obtained with NBD-phosphatidylglycerol followed by NBD-phosphatidylcholine.

Lipids 24, 691-699 (1989).

In a series of elegant papers, Pagano *et al.* (1-3) employed various NBD-labeled lipids and fluorescence microscopy to study the intracellular transport and metabolism of phospholipids in cultured animal cells. In 1984, Wittenauer *et al.* (4) reported that NBD-phospholipids could also be employed as fluorometric substrates in a convenient assay of lipolytic enzyme activity. Our laboratory has previously reported on the adaptation of this assay technique for several lipolytic enzymes from plant and fungal sources (5-8). In one of these studies (6) we reported that crude extracts from eight plant tissues were able to hydrolyze C₆-NBD-PC and that the rates of hydrolysis ranged from 0.021 to 4090 nmol/min/gram fresh weight of tissue. Although several other laboratories have reported on the use of either phospholipid or sphingolipid analogues which are labeled with fluorescent groups (9-13), the NBD technique which was described by Wittenauer *et al.* (4) appears to be the most convenient because this assay is continuous, and therefore does not

Abbreviations: BSA, bovine serum albumin; C₆-NBD or NBD hexanoic acid, 6-(7-nitro-1,3-benzoxadiazol-4-yl)amino]caproic acid; 4MUL, 4-methylumbelliferyl laurate; 4MUP, 4-methylumbelliferyl phosphate; NBD, 6-(7-nitro-1,3-benzoxadiazol-4-yl)amino; C₆-NBD-PC, 1-acyl-2-[6-(7-nitro-1,3-benzoxadiazol-4-yl)amino]caproyl phosphocholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PNP-PC, *p*-nitrophenyl phosphocholine; TAG, triacylglycerol.

require either lipid extractions or chromatographic separations. The present study was undertaken to evaluate the usefulness of the NBD technique to detect the activity of a diverse group of commercially available purified phospholipases and lipases.

MATERIALS AND METHODS

Materials. The lipase from *Rhizopus delemar* was obtained from Seikaguka Kogyo Co. C₆-NBD (the fluorescent cyclic free fatty acid), 4-methylumbelliferyl phosphate, *p*-nitrophenyl phosphocholine, and the other commercially prepared enzymes used in this study were obtained from Sigma Chemical Co. The NBD phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). These lipids are prepared from egg lecithin and contain a long chain fatty acid in the *sn*-1 position and an NBD-hexanoate or NBD-dodecanoate in the *sn*-2 position. The 4-methylumbelliferyl laurate (4MUL) was obtained from United States Biochemical Co. All other reagents were of the highest purity commercially available.

Enzyme assays. Phospholipase activity was measured using NBD-phospholipids as substrates by the procedure of Wittenauer *et al.* (4) with minor modifications. The reaction mixture (2 ml) contained 5 μM C₆-NBD-PC or other NBD lipid, 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.0) and 1 to 50 μl of enzyme sample. A standard curve of NBD-hexanoic acid concentration (0-7.5 micromolar) vs fluorescence was prepared by diluting 1 mM NBD-hexanoic acid (dissolved in ethanol) into the above 2 ml reaction mixture containing the concentrations of buffer and C₆-NBD-PC as stated above. This standard curve was used to convert changes in fluorescence to nmol of NBD-hexanoic acid produced. Relative fluorescence was measured continuously with a Sequoia-Turner Model 450 Fluorometer equipped with an excitation filter (460 ± 5 nm) and an emission filter (>535 nm). Esterase activity was measured using 4-methylumbelliferyl laurate (4MUL) as a substrate as previously described (14,15). The reaction mixture (2 ml) contained 50 mM potassium phosphate buffer (pH 8.0), 40 μl of 20 mM 4MUL in ethylene glycol monomethyl ether, and 1-50 μl of enzyme sample. The fluorometer was equipped with an excitation filter (360 ± 5 nm) and an emission filter (>415 nm). Phospholipase C activity was measured with PNP-phosphocholine (PNP-PC), essentially as described (16). The reaction mixture contained 1 mM PNP-PC, and 50 mM HEPES (pH 7.8) and 10-100 μl of enzyme sample. The increase in absorbance at 405 nm was measured with a recording spectrophotometer. Alkaline phosphatase activity was measured with 4-methylumbelliferyl phosphate (4MUP) using the same conditions as listed above for 4MUL except 4MUL was replaced with 4MUP.

Extraction, separation, and quantification of fluorescent products. A 2 ml reaction mixture containing C₆-NBD-PC was prepared as described above. After addition of enzyme, the mixture was incubated at 30°C. At various

time intervals the reactions were stopped by adding 200 μ l 1 M HCl. The lipids were immediately extracted by adding 6 ml 2:1 ethyl acetate:acetone. The lipids were removed in the upper organic phase and transferred to a clean test tube where the solvents were evaporated under a stream of N_2 . The lipids were redissolved in a small volume of 1:1 chloroform:methanol and spotted on a TLC (250 micron silica gel G) plate and developed in chloroform/methanol/30% ammonium hydroxide, 65/35/5. After development, the plates were thoroughly air dried and scanned for fluorescence with a Shimadzu Model CS-930 dual wavelength TLC scanner equipped with filter #3. The R_f values 0.27 and 0.18 for free C_6 -NBD and C_6 -NBD-PC, respectively, were determined by spotting samples of these compounds in separate lanes. The R_f values of 0.64 and 0.11 for NBD-diacylglycerol and lyso-NBD-PC, respectively, were deduced from the presence of unique products with phospholipase C and phospholipase A_2 , which corresponded well with previously published R_f values (1).

RESULTS AND DISCUSSION

Fluorescence of NBD-phospholipids and NBD-hexanoic acid under various conditions. In the first experiment the

relative fluorescence of C_6 -NBD-PC and its corresponding free acid, NBD-hexanoic acid or C_6 -NBD (the product of hydrolysis by A_2 -type phospholipases), were measured in two organic solvents, methanol and chloroform (Fig. 1) and in an aqueous solution buffered at pH 7.0 (Fig. 2). In chloroform and methanol the fluorescence of each compound was much higher than in the aqueous solution. In methanol the fluorescence of C_6 -NBD was about 70% higher than that of C_6 -NBD-PC. In chloroform, the fluorescence of C_6 -NBD was about 140% higher than that of C_6 -NBD-PC. In each organic solvent, the fluorescence was proportional to the concentration of either compound. In the aqueous solvent the fluorescence of C_6 -NBD-PC was very low, but was detectable and was concentration-dependent. Identical curves were obtained when the aqueous solutions were buffered with 50 mM citrate-KOH, pH 4.2 or 50 mM glycine-HCl, pH 9.0. The fluorescence of C_6 -NBD was much higher than that of C_6 -NBD-PC, but was about one-tenth of the value observed in methanol or chloroform (note scale change of abscissa). This experiment confirms the previous report of Wittenauer *et al.* (4) who observed that there was a 50-fold increase in fluorescence upon enzymatic removal of C_6 -NBD from C_6 -NBD-PC. An important finding of this experiment is that the fluorescence

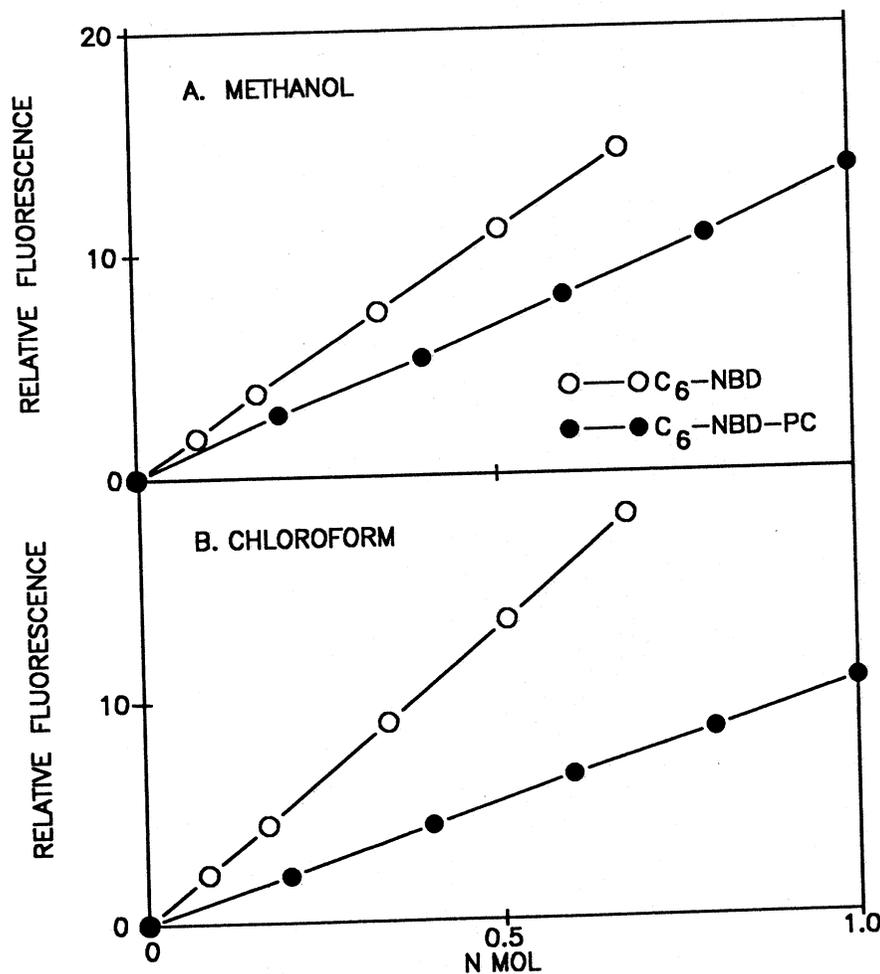


FIG. 1. The relative fluorescence of various concentrations of C_6 -NBD and C_6 -NBD-PC in methanol and chloroform.

of these two compounds is constant over the range of pH values of 5–9. This insensitivity to pH demonstrates that enzyme assays which employ NBD phospholipids as substrates can be buffered at any point in the physiological pH range. In contrast, several other spectrophotometric and fluorometric esterase substrates (i.e., *p*-nitrophenyl esters and 4-methylumbelliferyl esters) require alkaline pH values for maximum absorbance or fluorescence. There was no evidence of non-enzymatic hydrolysis of NBD lipids (increase in fluorescence) under any of the conditions which were investigated in this study.

In our initial studies, detergents and bovine serum albumin (BSA) appeared to interfere with the fluorescence of NBD lipids. Upon further examination, detergents or BSA induced a dramatic concentration-dependent effect on the fluorescence of C_6 -NBD-PC (Fig. 3). However, these compounds had no effect on the fluorescence of C_6 -NBD (data not shown). None of the five detergents which were studied had an effect on the fluorescence of C_6 -NBD-PC at detergent concentrations below 0.02 mg/ml. However, when the detergent concentration was increased, a unique point was reached for each detergent at which higher concentrations caused a significant increase in the fluorescence of C_6 -NBD-PC. These results suggest that the detergent concentration which starts to cause an increase in fluorescence of C_6 -NBD-PC may be correlated with the critical micelle concentration (CMC) of the detergent. A similar concentration-dependent effect was observed with BSA, with an increase in fluorescence occurring at concentrations of 0.05–0.1 mg/ml. This experiment indicates that most detergents and BSA should only be used with caution, and even then probably only within the lower concentration ranges described in Figure 3. If detergents had increased the fluorescence of the product, C_6 -NBD, then perhaps increased sensitivity could have been attained by including detergents in the

assay mixture, but because detergents only increased the fluorescence of the substrate, C_6 -NBD-PC, it is unlikely that detergents could provide any real advantage.

Hydrolysis of C_6 -NBD-PC by various phospholipases and lipases. In the original paper describing the use of C_6 -NBD-PC as a fluorometric substrate (4), it was used at a concentration of 5 μ M in an aqueous solution buffered at pH 7.0. We have utilized this convenient assay technique to measure phospholipase activities in crude extracts from a variety of plant tissues (5–8). In the next experiment, commercially prepared samples of five phospholipases and four lipases (Table 1) were tested for their ability to hydrolyze 5 μ M C_6 -NBD-PC. For each assay 1–100 μ g of protein was added to the cuvette and the enzyme activity was calculated from the mean of at least three different levels of protein that yielded product concentrations within the values shown in Figure 2 for a 5 min assay period. Increases in fluorescence were detected with the A_2 and C type phospholipases, and with each of the four lipases, but no increase in fluorescence was detected with the D type phospholipases. Among the enzymes which exhibited activity, the rates of hydrolysis ranged from 0.33 $\text{nmol min}^{-1} \text{mg protein}^{-1}$ for *R. delemar* lipase, to 1645.4 $\text{nmol min}^{-1} \text{mg}^{-1}$ for *N. naja* phospholipase A_2 assayed with Ca^{2+} . The phospholipase A_2 activities were dramatically stimulated by Ca^{2+} , which is characteristic of this group of enzymes. The activity of each of the other phospholipases (Table 1) was unaffected by Ca^{2+} . In order to compare this new assay technique with others commonly used for lipolytic enzymes, three other substrates were tested. With 4-methylumbelliferyl laurate, a common fluorometric esterase substrate, only the lipases exhibited activity. With *p*-nitrophenyl phosphocholine, a spectrophotometric substrate for phospholipase C activity, significant levels of activity were detected with the phospholipase C and a low

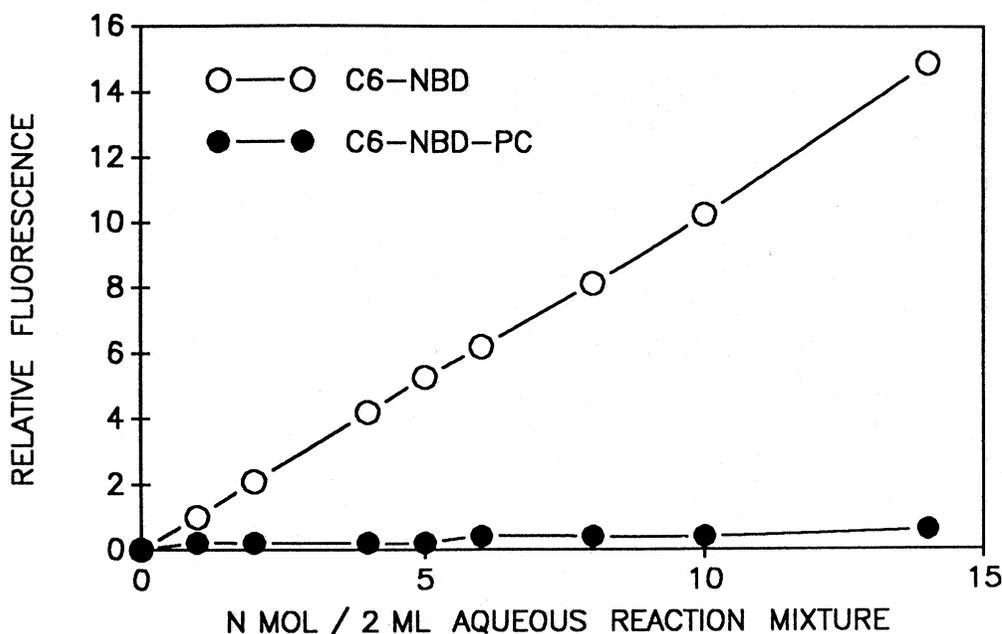


FIG. 2. The relative fluorescence of C_6 -NBD and C_6 -NBD-PC in 2 ml of an aqueous solution buffered with 50 μ M HEPES-NaOH, pH 7.0.

level of activity was detected with the phospholipase D from cabbage. With 4-methylumbelliferyl phosphate, a common fluorometric phosphatase substrate, activity was only detected with the phospholipase D from cabbage. It is not known whether this phosphatase activity is actually catalyzed by phospholipase D or is due to the presence of contaminating enzyme(s), because acid phosphatases are very common in plant tissues.

In order to compare the rates of hydrolysis of artificial substrates by these enzymes, the rates of hydrolysis of two natural substrates (PC for the phospholipases and TAG for the lipases) are also reported in Table 1. These

values were not determined in this study but were the specific activities that were reported by the suppliers of each of these commercially-prepared enzymes. It can be seen that for the two phospholipase A₂ enzymes, the rates of hydrolysis of the natural substrates was about 400 to 600-fold higher than the rates of hydrolysis of C₆-NBD-PC. With phospholipase C, PC appeared to be hydrolyzed at a rate that was 60,000-fold higher than that for C₆-NBD-PC, but the last section of this paper will present data which indicated that this value may not be reliable. For each of the lipases, TAG was the preferred substrate, followed by 4MUL, and finally by

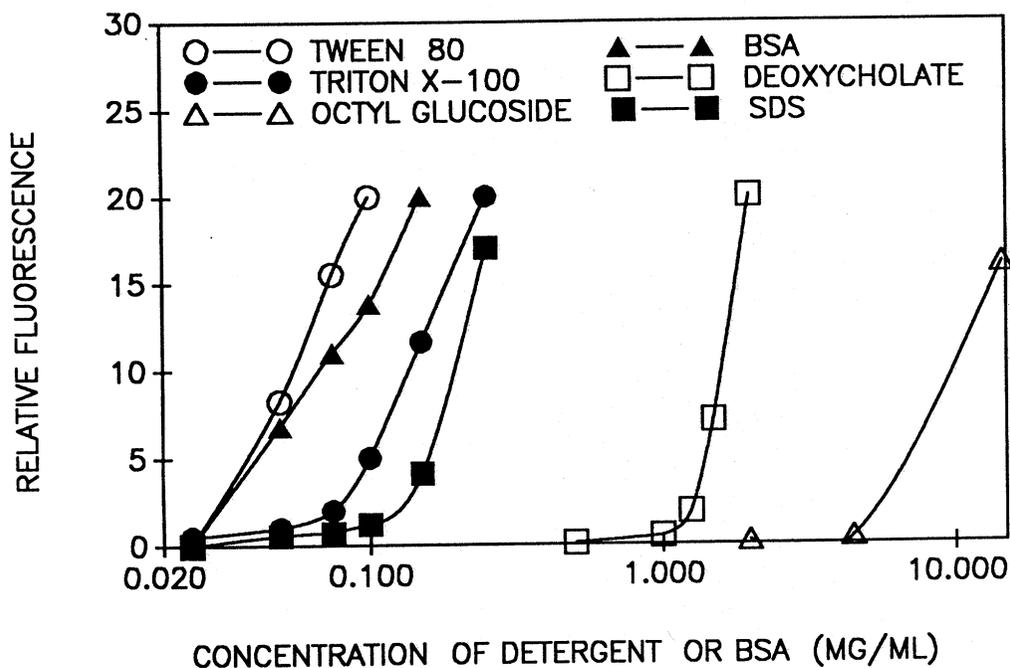


FIG. 3. Effect of various concentrations of detergents and BSA on the fluorescence of C₆-NBD-PC in an aqueous solution buffered with 50 μM HEPES-NaOH, pH 7.0.

TABLE 1

A Comparison of the Rates of Hydrolysis of C₆-NBD-PC and Other Common Substrates with Commercial Lipolytic Enzymes

Enzyme	Ca ²⁺ (3 mM)	Specific activity (nmol min ⁻¹ mg protein ⁻¹)					
		C ₆ -NBD-PC	4MUL	PNP-PC	4MUP	PC ^a	TAG ^a
Phospholipase A ₂ (<i>Naja naja</i>)	-	216	0	0	0	-	-
Phospholipase A ₂ (pancreatic)	+	1645.4	0	0	0	980,000	-
Phospholipase C (<i>Bacillus cereus</i>)	-	3.84	0	0	0	-	-
Phospholipase D (peanut)	+	156	0	0	0	600,000	-
Phospholipase D (cabbage)	-	19.2	0	80	0	1,160,000	-
Lipase (<i>R. delemar</i>)	+	18.3	0	74	0	-	-
Lipase (<i>R. arrhizus</i>)	-	0	0	0	0	10,000	-
Lipase (<i>C. cylindracea</i>)	+	0	0	0	0	-	-
Lipase (pancreatic)	-	0	0	1.24	60	9,000	-
Lipase (<i>R. delemar</i>)	+	0	0	0.87	56	-	-
Lipase (<i>R. delemar</i>)	-	0.33	1,471	0	0	-	600,000
Lipase (<i>R. arrhizus</i>)	-	198	34,470	0	0	-	6,000,000
Lipase (<i>C. cylindracea</i>)	-	1.06	4,360	0	0	-	7,000
Lipase (pancreatic)	-	2.33	194	0	0	-	2,000

^aRates of hydrolysis of phosphatidylcholine (PC) and triacylglycerol (TAG) as reported by suppliers of the commercially-prepared enzymes.

C₆-NBD-PC. This experiment demonstrates that NBD lipids may be useful as substrates for some lipolytic enzymes, especially for phospholipase A₂ activities, since none of the other three assays in Table 2 were capable of detecting A₂-type enzyme activity.

A comparison of the rates of hydrolysis of five NBD-phospholipids. In addition to C₆-NBD-PC, four other NBD lipids are commercially available. In the next experiment the activities of the three most active enzymes from the previous experiment were compared using the five NBD lipids (Table 2), each measured at a substrate concentration of 5 μM. With the *N. naja* phospholipase A₂ assayed in the presence of Ca²⁺, the highest activity was obtained with C₆-NBD-phosphatidylglycerol (C₆-NBD-PG), followed by C₁₂-NBD-PC, C₆-NBD-PC, C₆-NBD-phosphatidylethanolamine (C₆-NBD-PE), and finally C₆-NBD-phosphatidic acid (C₆-NBD-PA). With pancreatic phospholipase A₂ in the presence of Ca²⁺ the activity with C₆-NBD-PG was 14 to 25-fold higher than with the other four substrates. Finally, with the lipase from *R. arrhizus* measured in the absence of Ca²⁺, nearly equal levels of activity were obtained with C₆-NBD-PG, C₆-NBD-PE, and C₆-NBD-PC, and lower levels of activity were obtained with C₆-NBD-PA and C₁₂-NBD-PC. With the lipase, the activity with each of the substrates was inhibited by Ca²⁺.

Effect of concentration of NBD-lipids on rates of hydrolysis. In their initial description of the use of C₆-NBD-PC to assay enzymes, Wittenauer *et al.* (4) suggested that a concentration of 5 μM was saturating and would probably be optimum for most enzymes. In the next experiment the three enzymes which were studied in Table 2 were assayed with concentrations of C₆-NBD-PC and C₆-NBD-PG ranging from 1–20 μM (Fig. 4). With each of the three enzymes C₆-NBD-PG was hydrolyzed at a higher rate than C₆-NBD-PC. The reason that each enzyme hydrolyzed C₆-NBD-PG at a higher rate than C₆-NBD-PC is not known, but may have to do with a different aggregation state of the two lipids. Only with the lipase did the activity seem to become saturated with substrate and this occurred at about 10 μM. A similar trend was observed with C₆-NBD-PC except that the activities were consistently 20–30% lower than those observed with C₆-NBD-PG. This experiment indicates that for preliminary studies of the properties of an enzyme activity a 5 μM concentration of NBD lipid may be sufficient, but for more detailed kinetic analyses higher

concentrations of substrate may be necessary to ensure that saturating concentrations are achieved. Since Wittenauer *et al.* (4) reported the critical micelle concentration of C₆-NBD-PC to be 2×10^{-7} M, at a concentration of 5 μM greater than 90% of the C₆-NBD-PC would be present as aggregates or micelles. We have previously reported that two of the phospholipase activities in plants had Km values of 1.5 and 2.3 μM for C₆-NBD-PC (5). Each of the NBD-phospholipids was quite soluble in buffer solution except C₁₂-NBD-PC, which required shaking for at least 30 min to dissolve it. It should be noted that in this study the rates of hydrolysis of C₁₂-NBD-PC were estimated using the standard curve of C₆-NBD since C₁₂-NBD is not commercially available. We assume that this estimation should be fairly accurate since the fluorescence of C₁₂-NBD should be very similar to that of its C₆ analogue. We did purchase a methylated derivative of C₁₂-NBD, NBD-*N*-methylamino dodecanoic acid, but found that it exhibited very little fluorescence using the fixed excitation and emission wavelengths of this study. During these studies we observed that the fluorescence of each of the substrates appeared to decline gradually during several months even when stored in sealed glass ampoules at –10°C. The manufacturer reports a usable shelf life of 3–6 months in the freezer.

Separation of the products of hydrolysis of C₆-NBD-PC by TLC. In the final experiment (Fig. 5) the validity of measuring direct changes in fluorescence of the aqueous reaction mixture as a measure of activity was assessed by measuring the actual production of fluorescent products. After incubation with the enzymes, the lipids were extracted and separated by TLC and the levels of fluorescent product formed were quantified (4). For this experiment we chose to use C₆-NBD-PC as the substrate in conjunction with the two phospholipase A₂ enzymes and the phospholipase C enzyme. With the two phospholipase A₂ enzymes, there was a very good correlation between the rate of change of fluorescence of the entire solution and the rate of appearance of C₆-NBD. These results indicate that the continuous assay which we are describing is an accurate measurement of the enzyme activity of these two enzymes. In a previous study, Wittenauer *et al.* (4) reported that the levels of lyso NBD-PC were actually higher than the levels of C₆-NBD in similar time-course studies. In our hands there was a low level of fluorescent material which cochromatographed with lyso NBD-PC, but it only occurred in the 5 and 10 min

TABLE 2

A Comparison of the Rates of Hydrolysis of the Various C₆-NBD Phospholipids with Three Common Lipolytic Enzymes

Enzyme	Ca ²⁺ (3 mM)	Specific activity (nmol min ⁻¹ mg protein ⁻¹)				
		C ₆ -NBD-PC	C ₆ -NBD-PE	C ₆ -NBD-PA	C ₆ -NBD-PG	C ₁₂ -NBD-PC
PLA ₂ (<i>N. naja</i>)	–	216	621	516	172	466
	+	1645	563	360	5310	3040
PLA ₂ (pancreatic)	–	3.84	17.2	144	274	3.9
	+	156	138	145	2260	91.1
Lipase (<i>R. arrhizus</i>)	–	198	215	41.2	235	43.2
	+	145	97.4	25.9	89.4	22.3

^aEach substrate was tested at a concentration of 5 μM. The values reported are the mean of 3–5 determinations.

readings and was absent in the scans of extractions taken at later times in the time course (data not shown). This transient appearance of lyso NBD-PC was probably due to the presence of some *sn*-1-NBD-PC isomer. In this case the initial hydrolysis of non-NBD fatty acid from the *sn*-2 position (resulting in the appearance of lyso NBD-PC) is probably followed by hydrolysis of the C₆-NBD from the *sn*-2-NBD isomer. A similar phenomenon was observed

by Wittenauer *et al.* (4) except that their levels of lyso NBD-PC remained high throughout the experiment.

When the phospholipase C was examined the fluorescence of the aqueous solution increased at a nearly linear rate during the time-course (Fig. 5). However, upon examination of the reaction products by TLC, two important observations were made. The first was that there was no evidence of C₆-NBD at any of the sample times,

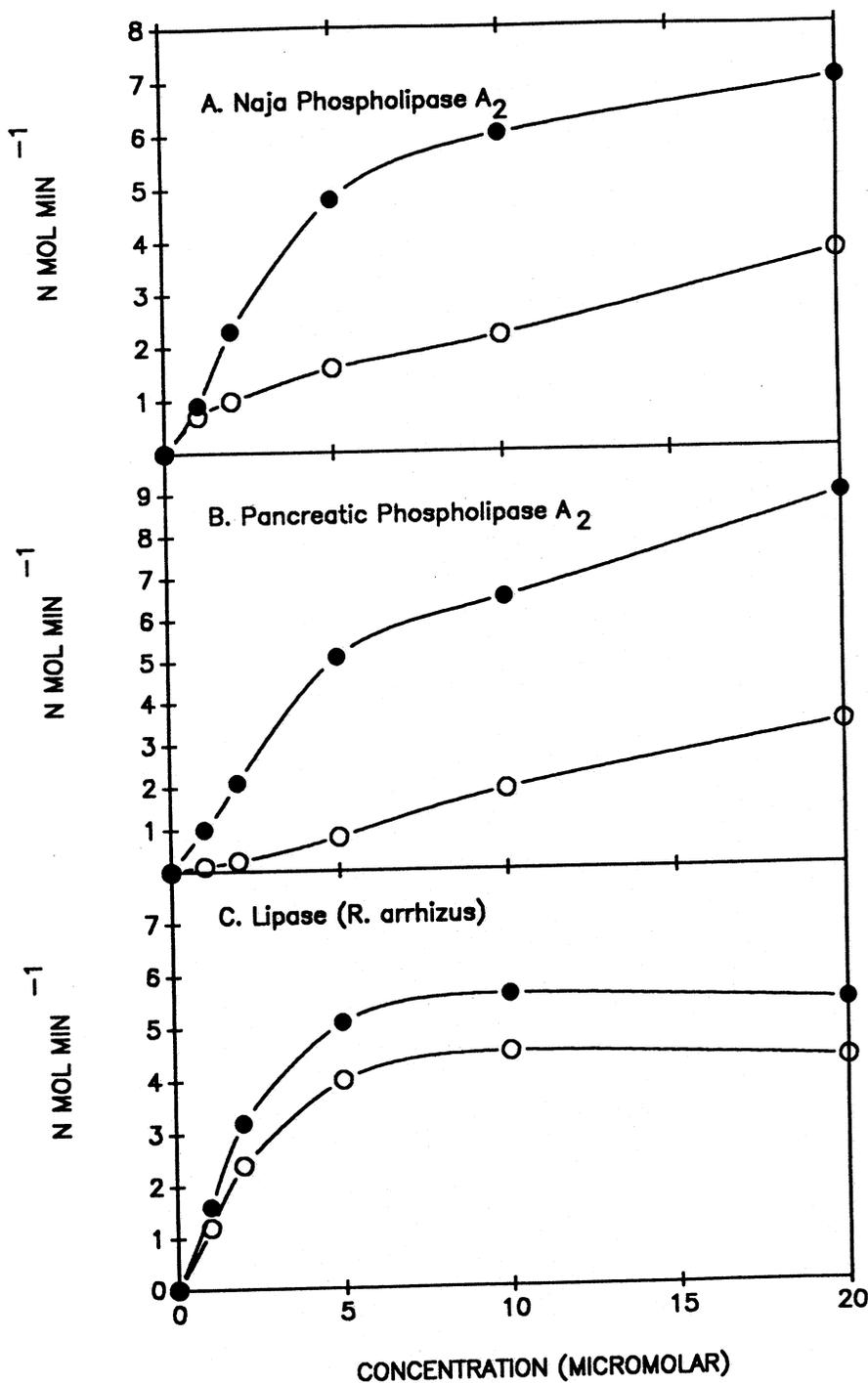


FIG. 4. Effect of concentration of NBD substrate on the enzyme activity of three commercial lipolytic enzymes. The symbols correspond to C₆-NBD-PG (●) and C₆-NBD-PC (○).

indicating that the increase in fluorescence of the solution was not caused by accumulation of the free acid and must be due to some other product. The second finding was that there was a rapid accumulation of C₆-NBD-diacylglycerol (DAG), the expected product of hydrolysis by C-type phospholipases. However, the accumulation of DAG began earlier and proceeded at a much faster rate than the increase in fluorescence of the entire aqueous

solution. This experiment indicates that although NBD lipids may be useful for detecting phospholipase C activity, the rates of increase in fluorescence of the reaction mixture may not be an accurate indication of the actual rates of formation of product. This experiment also indicates that one limitation of this assay technique is that it is incapable of distinguishing between phospholipase A₂ and C-type activities. These results differ from those

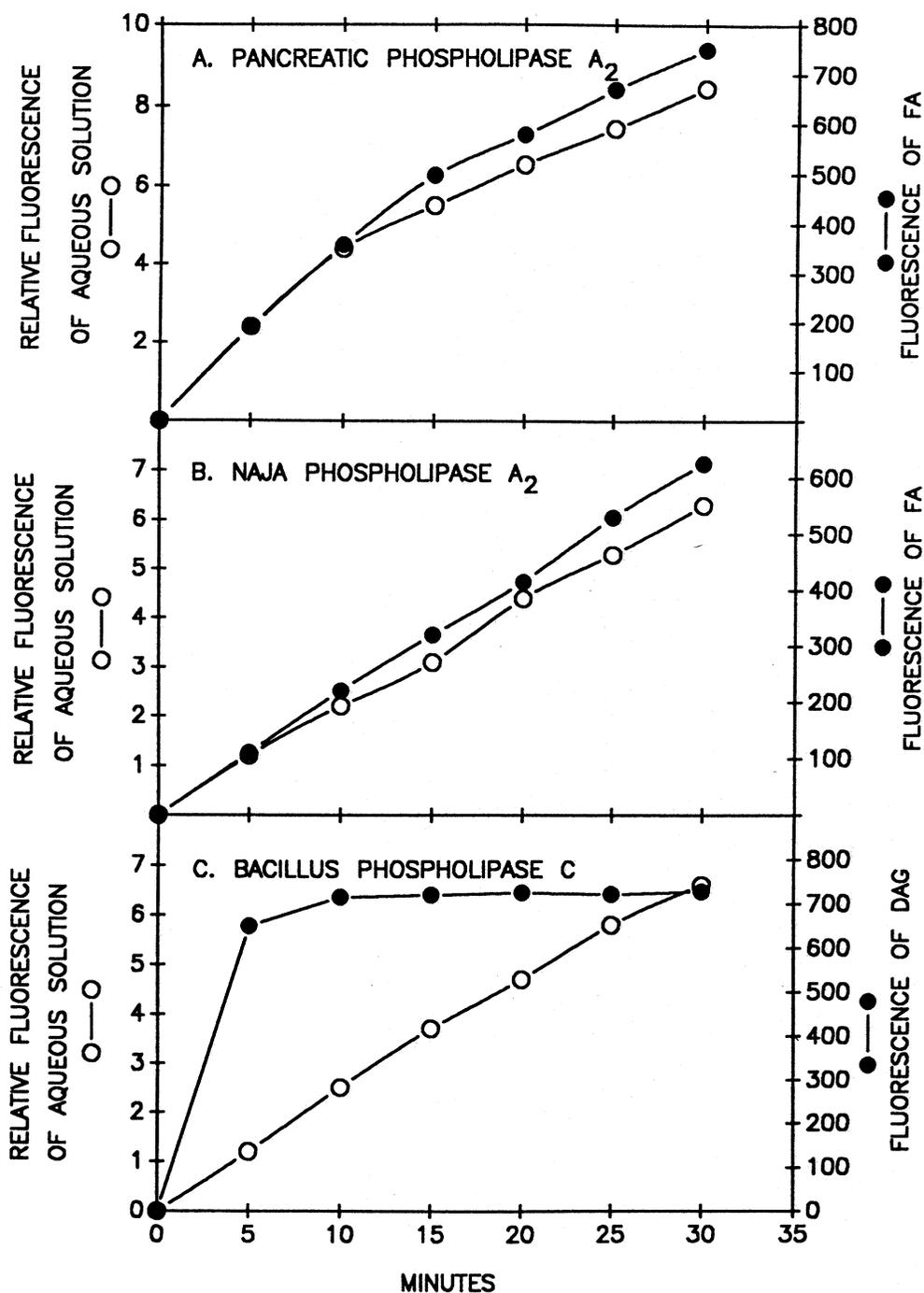


FIG. 5. Time-course study of the fluorescence of NBD-products (fatty acid [FA] or diacylglycerol [DAG]) measured after separation by thin layer chromatography, and the change in relative fluorescence of the aqueous reaction mixture.

of Wittenauer *et al.* (4) who reported that phospholipase C did not catalyze an increase in fluorescence when exposed to C₆-NBD-PC. Since they did not report the source of their phospholipase C it is possible that not all C-type phospholipases are capable of hydrolyzing NBD phospholipids.

With any new fluorometric assay technique there should be concern about the aggregation state of the substrate and product and possible binding of released product by proteins. The data presented in Figure 5 indicate that at least for the two phospholipase A₂ enzymes, these two potential problems did not occur. However, these problems may have accounted for the inaccurate detection of the phospholipase C activity. When applying this technique to a new enzyme or a crude enzyme mixture, it would be advisable to determine the products of hydrolysis chromatographically and correlate their rate of appearance with the rate of increase in fluorescence as was reported in this study.

CONCLUSIONS

The results of this study reveal that NBD phospholipids offer good potential as substrates for the assay of enzymes exhibiting phospholipase A₂ activity. They offer several advantages over more common methods for detecting this type of enzyme activity: 1) The assay technique described by Wittenauer *et al.* (4) and evaluated in this study is as sensitive as common techniques of measuring the production of the product of lipolytic reactions by radioisotopic or other analytical techniques (i.e., chemical analysis of fatty acid, choline, or glycerol production). 2) This assay technique is rapid and does not require any lipid extractions. Because each enzyme sample requires only 2-5 min per analysis, this technique is able to detect the types of rapid changes in enzyme activity that occur after treatment with various environmental or chemical stimuli. In two previous studies (5,7) we were able to employ the C₆-NBD-PC assay to measure the rapid and transient stimulation of a plant phospholipase activity upon treatment with calmodulin, protein kinases, and proteases. 3) This technique does not require sophisticated or expensive instrumentation. It can be performed using a simple and relatively inexpensive filter fluorometer. The instrument employed in this study is quite attractive because it uses a quartz halogen bulb which gives off very little heat. Therefore, the cuvette chamber does not need to contain a temperature control such as is required with fluorometers which utilize other types of bulbs. Although this study revealed that a filter fluorometer performed very nicely in the routine assays described in this study, it was unable to detect the small differences in the excitation or emission maxima which may have occurred in some of the experiments in this study. It is possible that the detergent effects which were observed in Figure 3 could be better explained by using a fluorometer with scanning capabilities. We have obtained very reproducible results using inexpensive disposable flint glass test tubes as fluorometric cuvettes. This is to be expected since both the excitation and emission wavelengths are in the visible spectrum. 4) All five NBD lipids used in this study are commercially available and reasonably priced. Since each assay only requires about 10 nmol of substrate, 10 mg of substrate is enough for

about 1000 assays. 5) An advantage which was previously mentioned is that this continuous assay can be conducted at any pH between at least 5 and 9 using the same standard curve (Fig. 2).

There are disadvantages to this assay technique, and it is important to be aware of the following: 1) For the two phospholipase A₂ enzymes in this study, the rates of hydrolysis of NBD phospholipids are several hundred-fold lower than the rates of hydrolysis of natural phospholipids. This could be a serious disadvantage for some applications. However, because the sensitivity of this assay (ability to detect minute levels of product formed) is several hundred-fold higher than that of most other techniques, this disadvantage may be cancelled out by its high degree of sensitivity. This technique may be especially useful for applications such as monitoring phospholipase A₂ activity during enzyme purifications. 2) As mentioned previously, this study revealed that this assay technique may not be able to distinguish between A₂ and C-type phospholipases. In this study, Ca²⁺ stimulated the A₂-type phospholipases but not the C-type. If this generalization can be shown to apply to other A₂ and C-type phospholipases, stimulation by Ca²⁺ may be a means of identifying the phospholipase A₂ activities. 3) Although C₆-NBD-PC is supposed to be supplied from the manufacturer with all of the C₆-NBD on the *sn*-2 position, a small amount of it appears to be esterified to the *sn*-1 position, as previously discussed. This problem of isomeric impurities needs to be acknowledged and may limit the usefulness of this lipid as a fluorometric substrate in certain applications.

Even though each of the lipases was capable of hydrolyzing C₆-NBD-PC (Table 1), there are at least two reasons why NBD-lipids are not the preferred substrate for studies with true lipases. First, each of the four lipases in this study hydrolyzed 4MUL at a high rate, suggesting that it is a better substrate for routine studies. Second, since the purity of these commercially-prepared lipases is questionable, we do not know whether their ability to hydrolyze NBD lipids is due to a broad specificity of the actual lipase molecule or is due to contamination by phospholipases(s).

REFERENCES

1. Pagano, R.E., Longmuir, K.J., and Martin, O.C. (1983) *J. Biol. Chem.* 258, 2034-2040.
2. Pagano, R.E., and Longmuir, K.J. (1983) *Trends Biochem. Sci.* 8, 157-161.
3. Pagano, R.E., and Longmuir, K.J. (1985) *J. Biol. Chem.* 260, 1909-1916.
4. Wittenauer, L.A., Shirai, K., Jackson, R.L., and Johnson, J.D. (1984) *Biochem. Biophys. Res. Commun.* 118, 894-901.
5. Moreau, R.A. (1986) *Plant Science* 47, 1-9.
6. Moreau, R.A. (1987) *Phytochemistry* 26, 1899-1902.
7. Moreau, R.A., and Morgan, C.P. (1988) *Plant Science* 55, 205-211.
8. Moreau, R.A. (1987) in *A.C.S. Symposium Series No. 325 Ecology and Metabolism of Plant Lipids* (Fuller, G., and Nes, W.D., eds.) pp. 343-354, American Chemical Society, Washington, DC.
9. Hendrickson, H.S., and Rauk, P.N. (1981) *Anal. Biochem.* 116, 553-558.
10. Gatt, S., Barenholz, Y., Goldberg, R., Dinur, T., Besley, G., Leibovitz-Ben Gershon, Z., Rosenthal, J., Desnick, R.J., Devine, E.A., Shafit-Zagardo, B., and Tsuruki, F. (1981) in *Methods in Enzymology* (Lowenstein, J.M., ed.) Vol. 72, pp. 351-375, Academic Press, New York.

NBD-PHOSPHOLIPIDS AS PHOSPHOLIPASE SUBSTRATES

11. Zeigler, M., Zlotogora, J., Regev, R., Dagan, A., Gatt, A., and Bach, G. (1984) *Clinica Chim. Acta* 142, 313-318.
12. Freeman, S.J., and Shankaran, P. (1985) *Can. J. Biochem. Cell Biol.* 63, 272-277.
13. Thuren, T., Virtanen, J.A., Lalla, M., and Kinnunen, P.K.J. (1985) *Clin. Chem.* 31, 714-717.
14. Guilbault, G.G., and Hieserman, J. (1969) *Anal. Chem.* 41, 2006-2009.
15. Roberts, I.M. (1985) *Lipids* 20, 243-247.
16. Kurioka, S., and Matsuda, M. (1976) *Anal. Biochem.* 75, 281-289.

[Received November 11, 1988; Revision accepted May 18, 1989]