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A continuous fluorescence-displacement assay for triacylglycerol lipase and phospholipase C that also allows the measurement of acylglycerols

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A new continuous fluorescence-displacement assay for enzymes that release long-chain fatty acids [Wilton (1990) Biochem. J. **266**, 435–439] is described in detail for pig pancreatic triacylglycerol lipase. The assay involves the displacement of the highly fluorescent fatty acid probe 11-(dansylamino)undecanoic acid from rat liver fatty acid-binding protein by long-chain fatty acids released as a result of enzyme activity. The assay is surprisingly effective for triacylglycerol lipase, allowing the expression of full activity with low concentrations of substrates in the absence of detergents. The initial rate of decrease in fluorescence is linearly related to enzyme concentration, and activity can be detected in the assay down to concentrations of 10 pg of pure enzyme/ml. The assays demonstrated the quantitative conversion of limiting amounts of substrate into the monacylglycerol. This observation allowed the assay to be used to measure substrates such as triacylglycerols and particularly 1,2-diacylglycerols at concentrations down to about 0.1 μM. Because phospholipase C releases 1,2-diacylglycerols, the coupling of this enzyme to excess lipase allowed the measurement of pure phospholipase C from *Bacillus cereus* at concentrations down to about 2 ng/ml, and the initial rate of fall in fluorescence in the assay was linearly related to enzyme activity.

INTRODUCTION

There is an increasing interest in lipases as industrial catalysts and as examples of enzymes that act at an oil/water interface. The recent description of the X-ray crystal structure of the acylglycerol lipases from *Mucor miehei* (Brady *et al.*, 1990) and human pancreas (Winkler *et al.*, 1990) will greatly facilitate studies on these enzymes. There is therefore an increasing requirement for a continuous, sensitive and versatile assay for these enzymes (Walde & Luisi, 1989).

Lipases are enzymes that normally hydrolyse neutral lipids to release long-chain fatty acids, and the present paper makes use of this general characteristic of lipases to allow a continuous measurement of enzyme activity in a competitive displacement assay. The assay system makes use of the fact that when the fluorescent fatty acid analogue 11-(dansylamino)undecanoic acid (DAUDA) binds to rat liver fatty acid-binding protein (FABP) there is a large fluorescence enhancement (up to 60-fold) as compared with the probe in buffer (Wilkinson & Wilton, 1986). The probe may be displaced competitively by low concentrations of normal long-chain fatty acids (Wilkinson & Wilton, 1987). The loss of fluorescence that results has already formed the basis of a continuous fluorescence-displacement assay for phospholipase A_2, and the general application of the assay to systems involving the release of long-chain fatty acids was highlighted (Wilton, 1990*a*).

In the present paper the application of the assay to triacylglycerol lipase from porcine pancreas is described in detail. The assay proved to be surprisingly efficient and was able to demonstrate the quantitative conversion of low concentrations of triacylglycerol (1–0.1 μM) into the expected monoacylglycerol. As a result it was possible, by using excess lipase, to quantify tri- and di-acylglycerols down to concentrations of the order of 100 ng/ml. In addition, because the system will rapidly hydrolyse 1,2-diacylglycerols to the monoacylglycerol, it has been possible to couple this assay to the release of 1,2-diacylglycerol from phosphatidylcholine catalysed by phospholipase C. As a result it was possible to produce a continuous fluorescence-displacement assay for the phospholipase C from *Bacillus cereus*.

EXPERIMENTAL

Enzymes

Purified pig pancreatic lipase (quoted specific activity 95600 μmol/h per mg), *Rhizopus arrhizus* lipase (quoted specific activity 405000 μmol/h per mg) and phospholipase C from *Bacillus cereus* (quoted specific activity 2500 μmol/min per mg) were obtained from Sigma.

Substrates and reagents

Highly refined olive oil suitable as lipase substrate, triolein, 1,2-dioleoyl-*sn*-glycerol and 1-stearoyl-2-arachidonyl-*sn*-glycerol were obtained from Sigma. Egg phosphatidylcholine and di-oleoylphosphatidylcholine were obtained from Lipid Products, South Nutfield, Surrey, U.K. DAUDA was obtained from Molecular Probes or synthesized by treating 11-aminoundecanoic acid with dansyl chloride. Rat liver FABP was prepared by methods previously described (Wilton, 1989).

Assay procedure

All assays were performed in 0.1 M-Tris/HCl buffer, pH 8, containing 0.1 M-NaCl as previously described (Wilton, 1990*a*), with the following modifications. Lipids were dissolved in ethanol at a concentration of either 2 or 10 mg/ml, and 0.1 ml of this solution was added to 20 ml of assay buffer, followed by 0.125 ml of 0.16 mM-DAUDA in methanol. Lower concentrations of lipid were obtained by mixing together the above assay cocktail with a cocktail without lipid in the appropriate proportions. FABP was added at a final concentration of 0.01 mg/ml. All assays were calibrated by the addition of up to 5 μl of 1 mM-oleic acid in methanol. Assays were performed at 25 °C.

Abbreviations used: FABP, fatty acid-binding protein; DAUDA, 11-(dansylamino)undecanoic acid.

RESULTS AND DISCUSSION

Assay of triacylglycerol lipase from pig pancreas

Normal conditions for the assay of pancreatic lipase involve presentation of high concentrations of the substrate in the form of an emulsion with detergents such as bile salts (Brockman, 1981). The continuous fluorescence-displacement assay already described in detail for phospholipase A_2 (Wilton, 1990*a*) can function effectively at low substrate concentrations, due to the sensitivity of the fluorescence method. Therefore it was decided to present the substrate (olive oil or triolein) as a simple 'microdispersion' by adding solutions of substrate in ethanol directly to the assay buffer in the absence of detergent. The final concentration of ethanol in the assay was routinely kept at 0.5% because excessive ethanol decreases the binding of ligands to FABP. At higher concentrations of olive oil the assay was noticeably turbid, but, due to the excellent spectral properties of DAUDA, the assay was not significantly affected.

In Fig. 1 the fluorescence-displacement traces are shown for pancreatic lipase over a 100-fold variation in concentration in order to illustrate the range and linearity of the assay. Of particular note is the sensitivity of the assay; it is possible to detect enzyme activity with picogram amounts of the purified enzyme. A minimal lag phase was observed with some assays, and hence rate measurements were taken at the point of maximal fluorescence change per minute. The linear response with enzyme concentration illustrated in Fig. 1 was very reproducible.

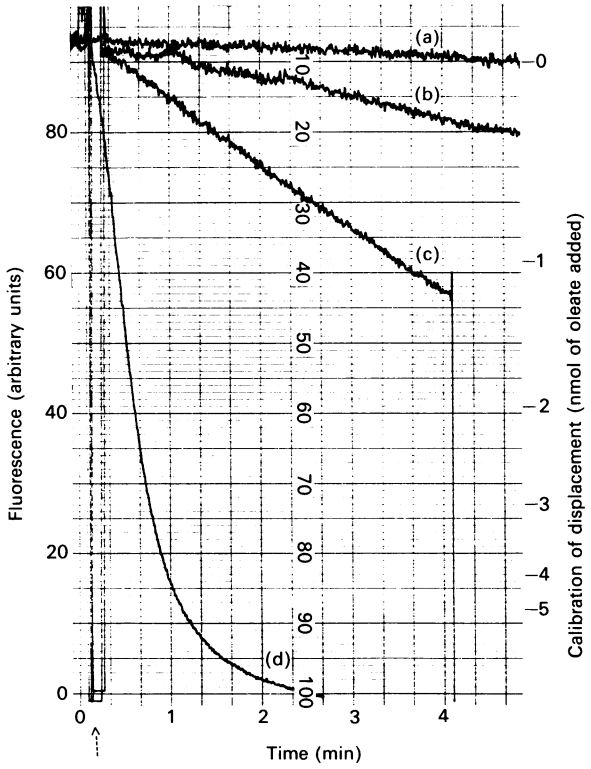

Fig. 1. Fluorescence-displacement traces resulting from hydrolysis of olive oil by pig pancreatic lipase

All assays (2 ml) contained 0.05 mg of olive oil/ml, 1 μM-DAUDA and 0.01 mg of FABP/ml. Trace (a) was with no addition of lipase, trace (b) was with 20 pg of enzyme, trace (c) was with 200 pg of enzyme and trace (d) was with 2 ng of enzyme. The point of addition of lipase is indicated by the arrow. Calibration of the fluorescence displacement by adding up to 5 μl of 1 mM-oleic acid in methanol to this assay is indicated.

When enzyme assays were performed in the absence of DAUDA and FABP, the linear rate of release of fatty acids was essentially identical with that when these components were present. Release was monitored by adding FABP and DAUDA after a certain assay time and comparing the fall in fluorescence with identical normal assays where DAUDA and FABP were present throughout the assay. This result clearly demonstrated that DAUDA and FABP did not have a significant inhibitory or stimulatory effect on the lipase assay.

Calibration of the assay by adding known amounts of oleic acid as a 1 mM solution in ethanol is also shown in Fig. 1. From the data in Fig. 1 the specific activity of the enzyme under the particular conditions of this assay may be calculated, and the average value from the three measurements is 1400 μmol/min per mg. This compares favourably with the value quoted for this sample of lipase of 1600 μmol/min per mg at 37 °C. A second sample of pancreatic lipase from the supplier (also quoted as 1600 μmol/min per mg) gave a value of 1700 μmol/min per mg in the fluorescence assay. Thus the ability to measure lipase activity at low substrate concentrations (50 μg/ml, nominally about 56 μM) allows the olive oil to be presented as a 'microdispersion' and results in a high expression of enzyme activity. The term 'microdispersion' is used as an operational description only, and does not suggest any particular physical state for the substrate.

The assay conditions for pancreatic lipase do not reflect the physical state of the lipid normally presented to the enzyme in the intestine and highlight the advantages of being able to assay insoluble non-polar substrates at low concentrations. Under these conditions the substrate must be sufficiently dispersed to provide an optimum surface area in the absence of added detergents. A similar phenomenon was observed with the assay of phospholipase A_2 (Wilton, 1990*a*), where again detergents were not required to demonstrate the expression of high enzyme activity.

Lipases are available from a number of micro-organisms, and the assay should be equally effective with these other sources of enzyme. This has been confirmed for the purified enzyme from *R. arrhizus*.

Measurement of the stoichiometry of the reaction catalysed by lipase using limiting concentrations of substrate

The effectiveness of the fluorescence-displacement assay for pancreatic lipase suggested that the assay should work well with limiting concentrations of substrate and provide a simple method not only of demonstrating the overall stoichiometry of the reaction but, in particular, for quantifying low concentrations of triacylglycerol. Therefore pancreatic lipase was assayed in the presence of various concentrations of olive oil down to a final concentration in the assay of 0.25 μg/ml. The range of concentrations used was achieved by mixing various proportions of assay cocktail containing 10 mg of olive oil/ml with assay cocktail without olive oil. Because the activity of the lipase varied linearly with substrate over the range of concentrations described in this paper, it was necessary to use a large amount of enzyme (10 μg) for reaction rates to be high enough to allow the study of reaction stoichiometry.

The results are shown in Fig. 2 and demonstrate a rapid reaction over 1–2 min, followed by a slow further fall in fluorescence that was significantly faster than the background rate (trace a) and increased with increasing concentration of substrate. Extrapolation of the slow phase back to zero time for each trace gave a value for the fall in fluorescence that could be quantified against the calibration of the experiment using oleic acid. Assuming that olive oil has a similar M_r to that of triolein, then the average value calculated from Fig. 2 was 2.01 ± 0.22 mol

of oleic acid equivalent released per mol of olive oil. This is in good agreement with the known stoichiometry of pancreatic lipase, which selectively hydrolyses the esters of fatty acyl groups with primary hydroxyl groups, giving the 2-monoacylglycerol product. The second phase of the reaction presumably reflects the slow hydrolysis of the 2-monoacylglycerol product.

Inherent in the data shown in Fig. 2 is the capacity of the assay to quantify samples of olive oil down to a concentration of about 20 ng/ml, and the initial fall in fluorescence was linearly related to lipid concentration provided that the total fall in fluorescence did not exceed about 50% (Fig. 3). Although the assay was quantified with oleic acid, all common long-chain fatty acids are equally effective at displacing DAUDA from FABP (Wilkinson & Wilton, 1987). It should be noted that FABP binds non-polar anions and no displacement of DAUDA was observed with 1,2-dioleoylglycerol or 2-mono-oleoylglycerol.

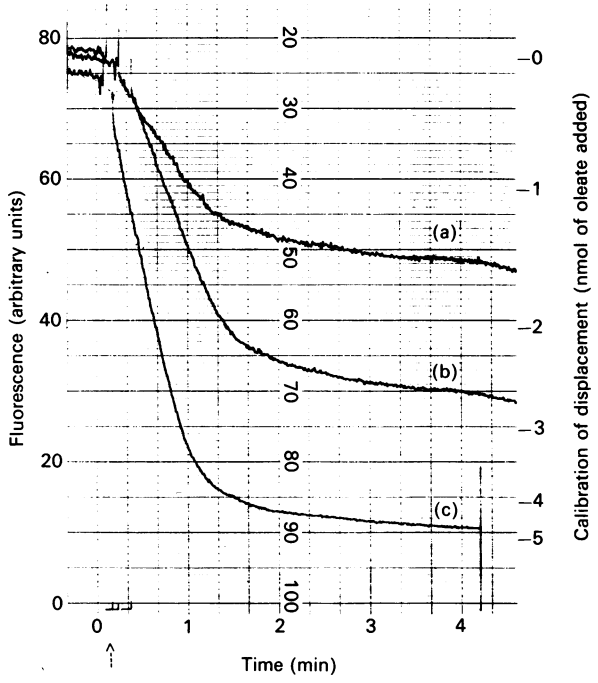

Fig. 2. Fluorescence-displacement traces resulting from the hydrolysis of limiting concentrations of olive oil by excess pig pancreatic lipase

All assays (2 ml) contained 10 μg of pig pancreatic lipase, 1 μM-DAUDA and 0.01 mg of FABP/ml. Trace (a) was with 0.25 μg of olive oil/ml, trace (b) was with 0.5 μg of olive oil/ml and trace (c) was with 1 μg of olive oil/ml. The point of addition of lipase is indicated by the arrow. Calibration of the fluorescence displacement by adding up to 5 μl of 1 mM-oleic acid in methanol to this assay is indicated.

Estimation of the acylglycerol 1-stearoyl-2-arachidonylglycerol

The capacity of pancreatic lipase to catalyse the rapid stoichiometric hydrolysis of substrate under the conditions of this fluorescence-displacement assay suggested that the method might be used to measure low concentrations of other long-chain acylglycerols containing an acyl chain at C-1 in addition to triacylglycerols.

Because hydrolysis of triacylglycerols to the corresponding 2-monoacylglycerol must proceed through the intermediacy of a 1,2-diacylglycerol, it should be possible to use the method to measure low concentrations of the important 1,2-diacylglycerols produced as a result of membrane phospholipid hydrolysis by phospholipase C. This possibility was investigated by using 1-stearoyl-2-arachidonyl-*sn*-glycerol, which is commercially available and is the physiologically important lipid product of phosphatidylinositide hydrolysis.

When standard amounts of 1-stearoyl-2-arachidonyl-*sn*-glycerol were titrated into the assay, a linear fall in fluorescence was obtained for up to 50% displacement, consistent with the normal displacement sensitivity of this type of assay (Wilton, 1990*a*). As shown in Fig. 3, the assay is linear with respect to fluorescence change over a range of substrate concentrations up to 500 ng/ml, with a lower limit of detection of about 50 ng/ml. Hence in a standard 2 ml assay 100 ng of 1-stearoyl-2-arachidonyl-*sn*-glycerol would be detected, and this brings the assay within the amounts of diacylglycerol released in physiological systems (Rittenhouse-Simmons, 1979). Such assays for diacylglycerols would require prior extraction and chromatographic separation of the diacylglycerols from other lipids, because the method would liberate fatty acids from other lipid classes due to the action of the pancreatic lipase.

1,2-Dioleoylglycerol was also evaluated in the assay and gave the expected stoichiometry and sensitivity.

Assay of phospholipase C using a coupled fluorescence-displacement assay linked to pancreatic lipase

The capacity of high levels of pancreatic lipase to convert 1,2-diacylglycerols quantitatively into the monoacylglycerol and fatty acid suggested that a coupled assay for phospholipase C

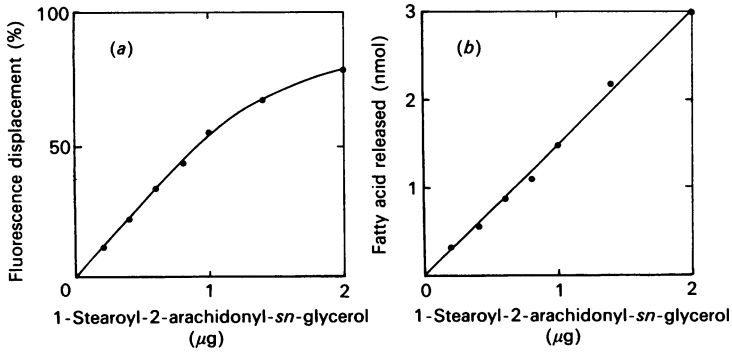

Fig. 3. Effect of 1-stearoyl-2-arachidonyl-*sn*-glycerol on fluorescence displacement when assayed in the presence of excess pancreatic lipase

All assays were as in Fig. 2. 1-Stearoyl-2-arachidonyl-*sn*-glycerol in ethanol (1–10 μl of 0.1 mg/ml) was titrated into the assay and the initial fall in fluorescence was determined. In (*a*) the initial fall in fluorescence is plotted against amount of diacylglycerol added to the assay, and in (*b*) the result is expressed in terms of nmol of oleic acid equivalents that are released. Values are the averages of three titrations.

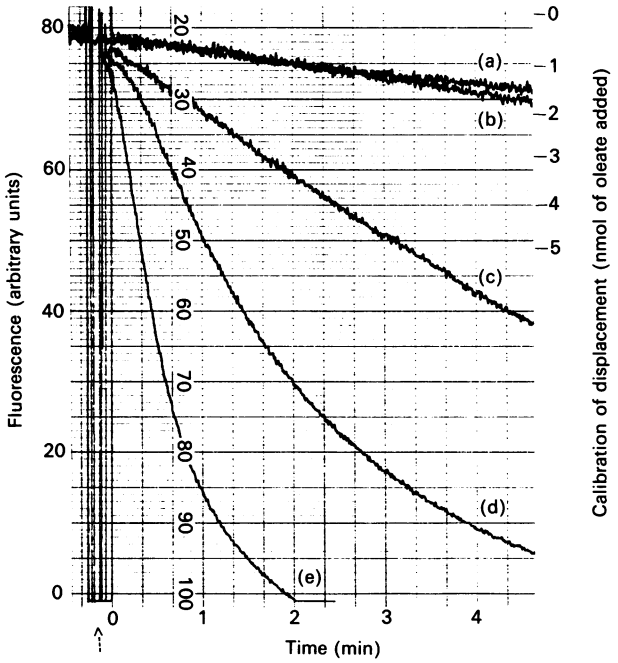

Fig. 4. Fluorescence-displacement traces resulting from the hydrolysis of dioleoylphosphatidylcholine by phospholipase C from *B. cereus* in the presence of excess pancreatic lipase

All assays (2 ml) contained 0.05 mg of dioleoylphosphatidylcholine/ml, 1 μM-DAUDA, 0.01 mg of FABP/ml and 10 μg of pancreatic lipase. Trace (a) was with no addition of phospholipase C; trace (b) was with 2 ng of enzyme; trace (c) was with 10 ng of enzyme; trace (d) was with 50 ng of enzyme; trace (e) was with 200 ng of enzyme. The point of addition of phospholipase C is indicated by the arrow. Calibration of the fluorescence displacement by adding up to 5 μl of 1 mM-oleic acid in methanol to this assay is indicated.

could be developed in which the diacylglycerol released by the phospholipase C was converted into monoacylglycerol by the lipase. The released fatty acid may then be monitored in a continuous fluorescence-displacement assay identical with that described above for lipase and for phospholipase A_2 (Wilton, 1990*a*).

Preliminary experiments with the purified phospholipase C from *B. cereus* established that such an assay was possible with egg phosphatidylcholine as substrate and high concentrations of pancreatic lipase. Although Ca^{2+} is normally included in phospholipase C assays (Little, 1981), this assay system had no requirement for Ca^{2+}, with essentially identical rates being obtained in buffers containing 5 mM-Ca^{2+} or with no Ca^{2+} and in the presence of 5 mM-EDTA.

The results of a typical assay using a range of phospholipase C concentrations are shown in Fig. 4 and demonstrate a linear relationship between initial rate and enzyme concentration. No significant activity was detected in the absence of phospholipase C, but a relatively high rate was obtained when the highly purified lipase from *R. arrhizus* was used instead of pancreatic lipase. This suggests that either this microbial enzyme preparation contains contaminating phospholipase A or C activity or that the enzyme has a broader specificity than the pancreatic enzyme and is able to demonstrate low activity towards this phospholipid. Hydrolysis of dipalmitoylphosphatidylcholine by this particular lipase has previously been demonstrated (Pluckthun & Dennis, 1982).

When high levels of phospholipase C (200 ng) were assayed in the absence of lipase, a significant rate was observed that showed an extended lag phase consistent with the phospholipase C preparation being contaminated with lipase activity. Lipase activity would allow the diacylglycerol formed to be slowly hydrolysed. This presumptive lipase activity would have no significant effect on the phospholipase C assays.

The calibration of the activity of this fluorescence-displacement assay by adding oleic acid gave values for the phospholipase C of 100 μmol/min per mg, as compared with the quoted value of 2500 μmol/min per mg with egg phosphatidylcholine at 37 °C. The relatively low activity expressed by this enzyme in the fluorescence-displacement assay probably reflects the low concentration of substrate that is used (0.07 mM) as compared with about 10 mM used for normal assays of this enzyme (Ottolenghi, 1969). The use of higher concentrations of phospholipid in the fluorescence assay results in a greater background fluorescence as more DAUDA partitions into the phospholipid vesicles with a resulting loss of sensitivity.

The assays shown in Fig. 4 were performed with dioleoylphosphatidylcholine as substrate. In order to evaluate the effectiveness of other substrates in the assay, particularly negatively charged phospholipids, the phospholipase C assay was performed with dioleoylphosphatidylglycerol. However, in this case a significant activity was observed simply on adding the lipase, and making it impossible to assay the phospholipase C. The ability of lipase to hydrolyse the primary ester bond of phosphoacylglycerols, albeit at a low rate, has previously been demonstrated (Slotboom *et al.*, 1970) and may limit the range of phospholipid substrates that can be used in this fluorescence assay.

General discussion

The assay for lipase described in this paper is surprisingly sensitive and allows the expression of high levels of lipase activity equivalent to that recorded in existing assays. The effectiveness of the basic assay for lipase must reflect the fact that the low concentrations of substrate used allow the substrate to be presented in the form of a 'microdispersion' and provides an optimal surface for enzyme binding and catalysis. Higher concentrations of substrate such as olive oil would eventually result in a coalescence of the dispersion to give oil droplets, with the resulting fall in surface area and a relative loss in enzyme activity. Normally high concentrations of substrate are dispersed with the aid of detergents such as bile salts.

The fluorescence-displacement assay for lipase has two particular advantages over the system for measuring phospholipase A_2. Firstly the dispersed substrate has minimal effect on the background fluorescence of DAUDA, thus improving the sample-to-background fluorescence-intensity ratio of the system. Secondly, with the release of excess fatty acids, there is complete displacement of DAUDA from FABP (see Fig. 1), and this is not the case for the phospholipase A_2 assay. This characteristic is at least partly explained by the absence of Ca^{2+} in the lipase assay. Ca^{2+} will complex with the released long-chain fatty acids and prevent an excessive rise in the concentration of the free anion required for DAUDA displacement. The assay of lipase in the presence of Ca^{2+} (2.5 mM) gave displacement properties similar to that observed for the assay of phospholipase A_2 (D. C. Wilton, unpublished work).

Although DAUDA binds with high affinity to albumin with an equivalent fluorescence enhancement to that shown for liver FABP, it does not bind to the primary long-chain fatty acid-binding sites on albumin. As a result albumin does not represent a sensitive alternative to liver FABP, because 3 mol of fatty acid have to bind to albumin before significant displacement of DAUDA occurs (Wilton, 1990*b*).

The requirement for FABP isolated from rat liver is a limitation

on the general usefulness of the assay. We have therefore undertaken a total chemical synthesis of the gene coding for rat liver FABP and overexpressed the active protein in *Escherichia coli* (A. F. Worrall, C. Evans & D. C. Wilton, unpublished work), so that it will become more readily available.

Like all indirect assays of product formation, the method may be compromised in very impure systems. In particular, systems containing membrane fractions or albumin greatly decrease the effectiveness of the fluorescence assay, and an initial purification step may be required to remove these components.

The generation of diacylglycerol as a result of phospholipase C-catalysed hydrolysis of phosphatidylcholine provided a method for the measurement of phospholipase C by the fluorescence-displacement assay. The assay is somewhat complex and must be used with particular caution when measuring impure biological samples. This is because of the necessity to use high levels of pancreatic lipase that will also hydrolyse other lipids present in the sample. However, the assay should be of use in monitoring enzyme purification and particularly in studying the properties of pure samples of phospholipase C in defined substrate systems.

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