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# Concurrent disappearance of *N*-acylethanolamine glycerophospholipids and phagolysosomes enriched in *N*-acylethanolamine glycerophospholipids as *Dictyostelium discoideum* cells aggregate

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**As the cellular slime mold, *Dictyostelium discoideum*, undergoes development, a phospholipid fraction containing 80% *N*-acylethanolamine glycerophospholipids (NAEGPs) and 20% acylphosphatidylglycerol (APG) disappears during the aggregation stage. In this study, the subcellular distribution of that NAEGP phospholipid fraction and the precise time period of disappearance of the fraction were determined. The content of the NAEGP fraction was determined in aggregating cells at 2-h intervals from the beginning of the developmental phase through 14 h, when the cells were completely aggregated. The NAEGP fraction comprised about 8% of the phospholipids in amoebae just starting the development cycle and about 12% in cells between 2 and 6 h of development; then its level decreased until it could not be detected at 12 and 14 h of development. The mole percentage of the total lipid phosphate in the NAEGP fraction was determined in isolated subcellular organelles. The phagolysosomes were enriched in the NAEGP fraction 1.7–2-fold over the level found in the amoebae and about 8-fold over the level in fractions highly enriched in the plasma membrane, mitochondria or peroxisomes. The content of phagolysosomes was determined by electron microscopy of aggregating cells. The amoebae contained large amounts of phagolysosomes up to 6 h of development, and then they gradually disappeared between 6 and 12 h of development. This combination of quantitative phospholipid analysis, subcellular organelle isolation and electron microscopy has revealed that in *D. discoideum* amoebae, the phagolysosomes were selectively enriched in the NAEGP fraction and both the NAEGP-enriched phagolysosomes and the NAEGPs disappeared concurrently between 6 and 12 h of development.**

## Introduction

When the vegetative amoebae of the cellular slime mold, *Dictyostelium discoideum*, exhaust their bacterial food supply, they commence a developmental stage by forming organized multicellular aggregates. During the aggregation process, a phospholipid fraction composed of 20% APG and

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Abbreviations: NAEGP, *N*-acylethanolamine glycerophospholipid; APG, acylphosphatidylglycerol; PNAE, phosphatidyl-*N*-acylethanolamine; PE, phosphatidylethanolamine.

80% NAEGPs disappears [1,2]. The NAEGPs [2–8] and APG [9–11] have been identified in other types of cells, but they seem to be related to lysosomes in certain cells. APG has been identified in whorl-filled liver lysosomes of chloroquine-treated rats [11] and in degenerating BHK cells which also contain whorl-filled lysosomes [10]. The NAEGPs comprise 26% of the phospholipids in granular cells of mammalian epidermis which are carrying out high levels of autophagy [6], and they have also been identified in degenerating BHK cells containing the whorl-filled lysosomes [10].

The amoebae cells of *D. discoideum* contain large amounts of whorl-filled phagolysosomes, and both the NAEGP-APG fraction [1] and the phagolysosomes [12] disappear during aggregation. This raised the possibility that the NAEGP-APG fraction (hereafter referred to as the NAEGP fraction) might be a component of the phagolysosomes, and as a result, both might disappear at the same time. To test this possibility, we have observed the disappearance of the NAEGP fraction and the phagolysosomes during aggregation. We have also determined the content of the NAEGP fraction in the phagolysosomes and some other subcellular organelles to see if the phagolysosomes were selectively enriched in the NAEGPs.

## Materials and Methods

*Growth and development of D. discoideum.* The following growth procedures have been described in detail [1]. *D. discoideum* amoebae were grown in association with a prototrophic strain of *Aerobacter aerogenes* on SM agar until they reached the end of exponential growth phase. Then the cells were harvested in cold water, separated from bacteria by centrifugation, and placed on 2% agar plates containing 0.04 M phosphate buffer (pH 6.8). After the cells had developed for a specified length of time on the agar plates, they were harvested in cold water, centrifuged at $200 \times g$ for 5 min, and stored at $-20°C$.

*Isolation of phagolysosomes.* Phagolysosomes were isolated by the sucrose density gradient centrifugation procedure used by Wiener and Ashworth [13].

First, a crude lysosomal-mitochondrial pellet was isolated, washed once with 0.25 M sucrose, resuspended in 0.25 M sucrose, and then layered on a linear 38–52% (w/v) sucrose gradient ($\rho$ = 1.16–1.25) mounted on a cushion of 61% (w/v) sucrose ($\rho = 1.30$). The gradient was centrifuged at $90\,000 \times g$ for 2.5 h in the small buckets of an SW 27 swinging bucket rotor in a Beckman Model L3-50 centrifuge. Fractions of constant volume were removed with a Buchler Auto Densi-Flow II C gradient remover and stored at $-20°C$.

*Enzyme assays.* Alkaline phosphatase (EC 3.1.3.1) is associated with the plasma membrane in *D. discoideum* [14] and was assayed as described by Loomis [15]. Succinate dehydrogenase (Ec 1.3.99.1) used as a marker for mitochondria was assayed as described by Bonner [16]. Acid phosphatase (EC 3.1.3.2) used to locate phagolysosomes was assayed according to Wiener and Ashworth [13]. Catalase (EC 1.11.1.6) used as a marker for peroxisomes was assayed as described by Aebi [17]. The protein concentration was determined by the method of Bradford [18] using the Bio-Rad Kit (Bio-Rad Laboratories, Richmond, CA).

*Isolation of the plasma membrane.* The plasma membrane was isolated using the poly(ethylene glycol)-Carbowax-Dextran 500 2-phase system of Brunette and Till [19] as it was previously used by Siu et al. [22] to isolate the plasma membrane from *D. discoideum* cells.

*Extraction and analysis of phospholipids.* Lipids were extracted from cells and subcellular fractions using $CHCl_3/CH_3OH$ (2:1, v/v) and $CHCl_3/CH_3OH$ (1:2, v/v) as previously described [1], except that the extraction of subcellular organelles with the $CHCl_3/CH_3OH$ (2:1) mixture was carried out for 3 h instead of 1.5 h. The phospholipid fraction was isolated by silicic acid column chromatography [1].

The amount of the NAEGP fraction was determined by quantitative thin-layer chromatography. The phospholipids were separated by silica-gel G thin-layer chromatography using $CHCl_3/CH_3OH/NH_4OH$ (40:10:1, v/v). The phospholipids on the chromatogram were detected by a phosphate-detecting spray [20], and the areas containing the phospholipids were scraped from the plate and analyzed for phosphate by the method of Bartlett [21], as previously described in detail [1].

*Electron microscopy of cells.* Cells were prepared for electorn microscopy by adding 5–10 ml 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) to cells developing on agar plates. After 60–90 min, the cells were scraped off the agar, centrifuged, washed twice with 0.1 M phosphate buffer (pH 7.4). The pellet of cells was resuspended in a small amount of warm agar. After cooling, the hardened agar-cell suspension was allowed to stand in 1% $O_SO_4$/0.05 M phosphate (pH 7.4) for 45–60 min, and then it was dehydrated serially for 2 min with cold 30%, 50%, 70%, 90% ethanol in water. Next it was allowed to stand in 100% ethanol for 10 min at room temperature before being washed twice with propylene oxide. It was hardened in propylene oxide/Epon 1 : 1 solution for 2 h and then embedded in Epon. The thin sections were stained with uranyl acetate and lead citrate and were scanned for lamellar-filled phagolysosomes using an RCA-3G electron microscope.

## Results

*Disappearance of the NAEGP fraction during development*

When the amoebae were placed on agar, they commenced the developmental program during which they aggregated and eventually formed fruiting bodies composed of stalks and spores. Cells were harvested from the agar at 2-h intervals and analyzed for the content of the NAEGP fraction to determine when the lipid fraction disappeared. In the one-dimensional thin-layer chromatography system used, the NAEGP fraction had an $R_f$ value of 0.7 and was separated from all other phospholipids, making it a system to conveniently and rapidly determine the mol% of the total lipid phosphate present in the NAEGP fraction. The results of the phospholipid analyses of cells at different stages of development are presented in Fig. 1. During the first 6 h of development, the cells maintained high level of the NAEGP fraction. After 6 h of development, the content of NAEGPs began to decline until there was none detectable at 14 h, a result in agreement with our previous study which measured the NAEGP content at 4 and 14 h of development [1].

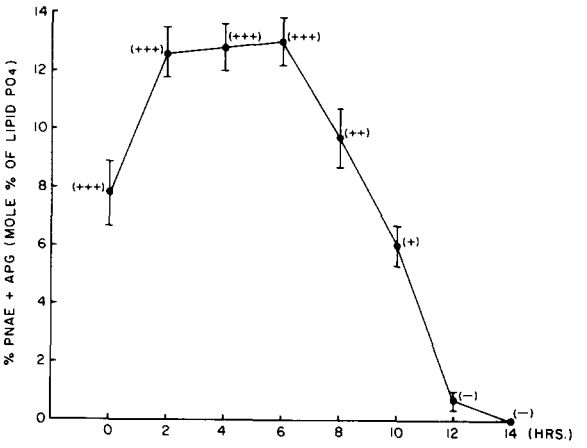


Fig. 1. Disappearance of the NAEGP-APG phospholipid fraction and phagolysosomes during development. Amoebae were placed on buffered agar and allowed to develop for the time specified. They were harvested and analyzed for the amount of the NAEGP-APG fraction or the content of whorl-filled phagolysosomes. The mol% of the NAEGP-APG fraction (●———●) and the relative content of phagolysosomes (+ + +, maximum; −, none) are shown for the specified times of development. The mol% of the lipid fraction is presented as the mean ± standard deviation. The relative content of phagolysosomes was determined visually by noting numbers of whorl-filled phagolysosomes in a cell and the frequency of thin sections containing phagolysosomes. The maximum amount (+ + +) was 3–4 phagolysosomes per cell and that content was in every thin section examined. Lower numbers of phagolysosomes are indicated by (+ +), and (+) (see text for details). The morphological forms of the developing cells were: smooth lawn of cells from 0–6 h; rippled lawn at 8 h; elongated aggregates at 10 h; mounds at 12 h; mounds with pointed tips at 14 h.

*Disappearance of phagolysosomes during development*

When amoebae cells which had undergone development for 0–6 h were examined by electron microscopy, they contained a large number of phagolysosomes filled with lamellated inclusion bodies or whorls. An electron micrograph showing the whorl-filled phagolysosomes in a typical cell which had undergone development for 4 h is shown in Fig. 2, and it shows that the phagolysosomes are easily recognized, so their presence can be easily detected in cells. Electron micrographs were made at 2-h intervals from cells which had just commenced development (0-h cells) through 14-h-aggregated cells, and the disappearance of the

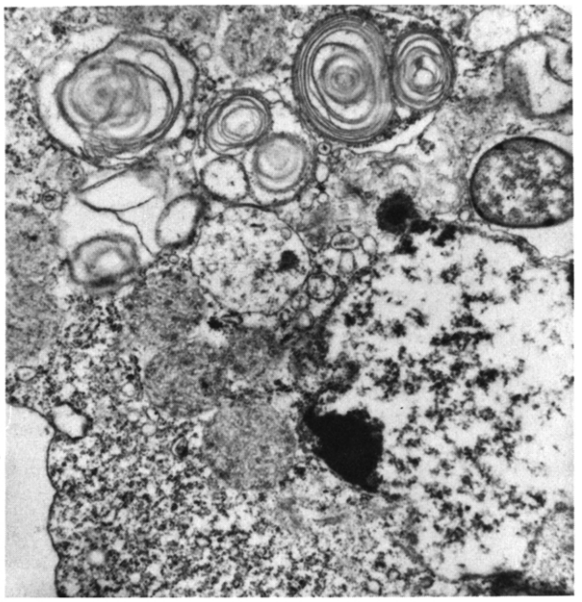

Fig. 2. Electron micrograph of a *D. discoideum* cell which had developed for 4 h. The phagolysosomes filled with lamellated residual bodies are prominent. Magnification, ×20480.

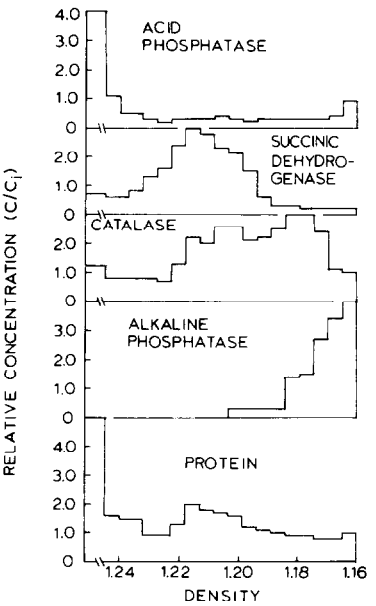


Fig. 3. Distribution of protein and enzyme activities after sucrose density gradient centrifugation of the crude mitochondrial-lysosomal pellet. See the text for details of centrifugation techniques. The interruption of the abscissa represents the change from the cushion ($\rho = 1.3$) to the maximum density in the gradient ($\rho = 1.25$).

whorl-filled phagolysosomes as the cells undergo development is presented in Fig. 1. By examining the electron micrographs (not presented), it was easily seen that in the cells which had developed up to 6 h, every thin section of a cell examined contained 3–4 whorl-filled structures (indicated by + + + in Fig. 1). In cells which had developed for 8 h, not all cell thin sections contained the whorl-filled phagolysosomes, indicating that they had started to disappear (indicated by + + ). By 10 h, most of the cells had no phagolysosomes, and many fields had to be scanned before any could be found (indicated by +). In 12- and 14-h cells, no phagolysosomes were observed (indicated by −).

*Subcellular distribution of the NAEGP fraction*

When the crude lysosomal-mitochondrial pellet was subjected to centrifugation in the sucrose density gradient, the observed relative distribution of protein and enzymes shown in Fig. 3 were similar to those found by Wiener and Ashworth [13]. The recovery of protein and total activity of the enzymes shown in Fig. 3 from the gradient was 95% or greater, compared to the amount layered on the gradient. The acid-phosphatase-enriched phagolysosomes collected at the interface between the 61% sucrose cushion and the gradient, and they also traveled into the cushion. There was no enrichment of marker enzymes for the other subcellular organelles in the phagolysosome fraction. The acid phosphatase activity was found to be partially latent, because its activity could be doubled by adding 0.1% Triton X-100 to the assay mixture or by freezing and thawing the organelles. Fractions of density of 1.24 and greater, including the cushion, were collected and used for lipid extraction of phagolysosomes.

The top of the gradient was enriched in the plasma membrane enzyme, alkaline phosphatase, but we did not use this fraction for phospholipid extraction, because it was not well-characterized. Instead, we isolated a plasma membrane fraction by a method which Siu et al. [22] had previously shown would yield a highly purified plasma membrane fraction from *D. discoideum.* Our plasma membrane fraction had a 16-fold enrichment of the *D. discoideum* plasma membrane enzyme, alkaline phosphatase, over the homogenate, an en-

richment slightly higher than the 12-fold enrichment obtained by Siu et al. [22]. The plasma membrane fraction had little or no enrichment in acid phosphatase (for phagolysosomes), succinate dehydrogenase (for mitochondria) or catalase (for peroxisomes). Since our results were similar to those of Siu et al., our plasma membrane fraction was probably the same as their well-characterized one, and we used it to determine the level of NAEGPs in the plasma membrane.

The content of the NAEGP fraction in the whole cells and subcellular organelles was determined by extracting the phospholipids and separating them in a thin-layer chromatography system which separated that fraction from all others. The percent of the NAEGP fraction was determined as an individual thin layer fraction; all other phospholipids were not separated but were analyzed as groups of phosphate-containing lipids. The mol% of the total lipid phosphate in the NAEGP fraction was $5.5 \pm 0.2$ in the amoebae used, the value being the mean $\pm$ the standard deviation ($n = 6$ or more). The phagolysosomes isolated from those amoebae contained $11.5 \pm 0.2$ mol% NAEGP, but a mitochondrial-peroxisome fraction and the plasma membrane each contained only about $1.5 \pm 0.1$ mol%. When organelles isolated from seven gradients and six plasma membrane preparations were analyzed, the phagolysosomes were routinely enriched 1.7–2-fold in the NAEGP fraction compared to the amoebae and 7–8-fold compared to the plasma membrane, mitochondria and peroxisomes.

## Discussion

A good system to study the metabolism of membrane phospholipids would be one in which a particular phospholipid is specifically associated with a subcellular organelle and both the phospholipid and organelle disappear concurrently. Such a system would offer the unique opportunity to investigate the factors which regulate the formation and degradation of the organelle in terms of enzyme activities which synthesize and degrade the phospholipid.

Our results obtained from the combination of quantitative phospholipid analysis, subcellular organelle isolation, electron microscopy indicate that in *D. discoideum* the NAEGP is a specific phagolysosomal lipid, and both the phospholipid and the organelle disappear concurrently. We have already obtained evidence suggesting that there are enzymes which specifically degrade NAEGPs, because the lipid contains the *N*-acylethanolamine bond. We have detected phospholipases $A_1$ and D which hydrolyze PNAE at low pH values and a phospholipase $A_2$ which hydrolyzes phosphatidylethanolamine but not phosphatidyl-*N*-acylethanolamine at pH 7.2 [25], demonstrating that at least one lipid-metabolizing enzyme in *D. discoideum* does recognize the presence of the *N*-acyl bond. We are currently measuring the phospholipase activities which hydrolyze PNAE during the aggregation stage to learn whether an increase in hydrolytic activity is partialy responsible for the disappearance of the NAEGPs.

Granular epidermis cells [6] and degenerating BHK cultured cells [10] contain NAEGPs and have high levels of autophagy, a process associated with secondary lysosomes, so it is possible that the NAEGPs are also associated with secondary lysosomes in those cells. The other phospholipid in the *D. discoideum* NAEGP fraction, APG, has been shown to be a component of liver lysosomes in chloroquine-treated rats, suggesting that APG may also have some lysosomal function in certain cells.

We have found that in the pH range of 3.8–4.5, PE is hydrolyzed to water-soluble components by a *D. discoideum* homogenate, conditions under which PNAE is hydrolyzed to lyso-PNAE and *N*-acylethanolamine is released at 0.1 the rate of lyso-PNAE formation [25]. It may be that the NAEGPs help protect the lysosomal membrane under conditions where extensive phospholipid hydrolysis is occurring. While the common glycerophospholipids are being completely hydrolyzed within the lysosome, the NAEGPs seem to be resistant to complete hydrolysis, and may, thereby, help maintain the structural stability of the membrane.

When the amoebae phagocytose bacteria, the portion of the membrane surrounding the bacteria is internalized, and surface area of the internalized membrane is 40–50% of the plasma membrane [23,24]. As the amoebae cells aggregate, the whorl-filled phagolysosomes move to the cell surface, fuse with the plasma membrane, and re-

lease their contents by exocytosis [12]. Since the internalized phagolysosomal membrane is recycling to the plasma membrane and since we found that the percentage of the NAEGP fraction in the phagolysosomes was 7–8-times higher than that in the plasma membrane, it appears that the NAEGPs are not becoming part of the plasma membrane as the phagolysosomes fuse with it during exocytosis of the whorls. This might be a system in which the metabolism of the unique secondary lysosomal phospholipid could be specifically investigated during the membrane-recycling process.

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