Activated neutrophils release a variety of eicosanoids into the extracellular medium including arachidonic acid, 5-hydroxyicosatetraenoic acid, and leukotriene A\textsubscript{4} and B\textsubscript{4}. In this study, the mechanism of arachidonic acid export has been examined using inside-out plasma membrane vesicles from pig polymorphonuclear leukocytes. Tritiated arachidonic acid associated rapidly with the membrane vesicles and crossed the membrane into the intravesicular space in a time-dependent and saturable manner. Half the maximal influx rate was measured at an arachidionate concentration of 5.7 \(\mu\)M, and a maximal influx velocity of 3.0 nmol/mg \(\times\) min was determined at pH 6.8. Influx into vesicles was sensitive to a number of common anion transport inhibitors including pentachlorophenol, phloretin, diiodosalicylic acid, and quercetin as well as to the proteases trypsin and Pronase, suggesting a protein-dependent process. Furthermore, influx was temperature-sensitive with an energy of activation of 11.6 kcal/mol. Varying extravesicular concentration of ATP, Na\textsuperscript{+}, or K\textsuperscript{+} had no impact on arachidionate influx, whereas changes in pH had a profound effect; optimum transport activity was observed at an extravesicular pH of 6, whereas raising the pH to 9.5 essentially abolished uptake. These results indicate and initially characterize a novel protein-facilitated arachidionate export mechanism in pig neutrophils.

Polymorphonuclear leukocytes (PMN\textsuperscript{1}) are the predominant cell type present in areas of acute inflammation. Activated neutrophils release a number of eicosanoids such as arachidonic acid (1–3), 5-hydroxyicosatetraenoic acid, and leukotriene A\textsubscript{4} (4) and B\textsubscript{4} (5) into the plasma where they may amplify or perpetuate the acute inflammatory response. Recent progress in leukotriene research has led to a more detailed understanding of the molecular biology and enzymology of the oxidative metabolism of arachidonic acid and to the development of potent inhibitors of biosynthesis as well as receptor antagonists interfering with signal transduction (6, 7). The mechanism of how eicosanoids, once biosynthesized, leave the producing cell to reach their target cell is, however, still poorly understood. Recently, an ATP-driven export pump for cysteinyl leukotrienes has been identified in hepatocytes (8), erythrocytes (9), and heart cells (9, 10). In addition, in intact PMN a LTB\textsubscript{4} export carrier has been identified and characterized (11). To study the cellular export mechanism, whole cell systems are too complex to allow the accurate measurement of transport kinetics and of the effects of inhibitors. Furthermore, the situation for PMN is particularly complex since these cells do not only biosynthesize and release eicosanoids but also avidly take up and metabolize arachidonic acid, LTA\textsubscript{4} (1, 4), and LTB\textsubscript{4} (12).

To overcome these restrictions imposed by whole cell systems to the investigation of transport mechanisms, we prepared inside-out plasma membrane vesicles from PMN and investigated the usefulness of this system to study eicosanoid transport using arachidonic acid as a ligand. Inside-out plasma membrane vesicles without contamination with right side-out vesicles were prepared by isolating phagocytic vesicles from PMN, which have engulfed particles, the ingested material is encased within a vesicle derived from the plasma membrane with an inside-out orientation (13). After phagocytosis of albumin-coated mineral oil droplets, those phagocytic vesicles can readily be isolated and have been used for Ca\textsuperscript{2+} transport studies (14).

The mechanism of the translocation process of fatty acids through the plasma membrane in general is controversial (15). On one hand, it is argued that fatty acid movement through the phospholipid bilayer is an entirely unregulated process in which diffusion of fatty acids in either direction of the membrane is driven by concentration gradients (16). Juxtaposed to the diffusion hypotheses, there is increasing evidence for a facilitated protein-dependent transport pathway for long chain fatty acids. Five putative fatty acid transport proteins have been identified in the plasma membrane of several tissues including adipocytes, liver, endothelium, brain, intestine, kidney, lung, skeletal, and cardiac muscle. For three of these cDNA clones have been isolated (reviewed in Ref. 15). Schaffer and Lodish (17) first succeeded not only in expression cloning and purification of one putative carrier protein but could also reconstitute transport function, thus directly demonstrating its physiological role.

The results reported herein meet several criteria for a protein-facilitated export mechanism for arachidonic acid in PMN plasma membrane vesicles, such as substrate saturation, inhibition by a variety of anion transport inhibitors (18), and sensitivity toward proteases. The ionic mechanism seems to be that of an H\textsuperscript{+} + arachidonate cotransport system operating with an energy of activation of 11.6 kcal/mol. With the transport assay presented here, it may be possible to separate the transport processes out of the complex cascade of events starting after activation of PMN by various stimuli to study the mechanism(s) of putative eicosanoid transporters and to investigate the impact of leukotriene inhibitors on transport.

\textsuperscript{1} The abbreviations used are: PMN, polymorphonuclear leukocyte(s); LT, leukotriene; PIPEP, piperoxane-N,N’,N’-bis(2-ethanesulfonyl) acid; BSA, bovine serum albumin; FCP, pentachlorophenol; PL\textsubscript{A}2, phospholipase A\textsubscript{2}; LXA\textsubscript{4}, lipoxin A\textsubscript{4};
MATERIALS AND METHODS

Biochemicals—Mineral oil (M-5904), lipopolysaccharide O26:B6 (L-3755), phenylmethylsulfonyl fluoride, Triton X-100, and PIPES di-tpassium salt were from Sigma. Ficol-Paque, dextran T-500, and ConA-Sepharose were obtained from Pharmacia Biotech Inc. Arachidonic acid was from Fluka, and [5,6,8,9,11,12,14,15-3H]arachidonic acid (230.5 Ci/mmol) was purchased from DuPont NEN. Fatty acid-free bovine serum albumin was from Boehringer Mannheim.

Pig Polymorphonuclear Leukocytes (PMN)—Arterial blood was collected from 3- to 4-month-old pigs using sterile Na2EDTA (2.14 g/liter) as an anticoagulant. The whole blood was centrifuged at 200 × g for 15 min, and the platelet-rich supernatant was discarded. PMN were prepared by dextran sedimentation, hypotonic lysis of remaining erythrocytes, and centrifugation on Ficol-Paque, as described (19).

Purification of Phagocytic Vesicles by ConA Chromatography—Purified PMN (50 to 106/ml) were resuspended in modified Hank’s buffer (124 mM NaCl, 4.9 mM KCl, 0.66 mM NaH2PO4, 0.64 mM KH2PO4, 15 mM NaHCO3, 0 mM Tris-HCl, pH 7.4) and incubated with a mineral oil emulsion, essentially as described (13). Briefly, 1 volume of mineral oil and 4 volumes of modified Hank’s buffer containing 20 mg/ml fatty acid-free albumin were mixed and sonicated in a Branson Sonicator (Cell Disruptor B15), with a microtip for 2 × 1 min, setting 5. The oil emulsion was centrifuged for 5 min at 1600 × g, and the thin layer of uncovered droplets at the surface was removed. The remaining stable emulsion was mixed with 1 volume of fresh pig serum. One volume of this oil emulsion was added to 10 volumes of PMN suspension at 4 °C. Phagocytosis was initiated by the addition of 0.9 mM CaCl2, 1.27 mM MgCl2, and 10 ng/ml lipopolysaccharide and warmed to 37 °C. After 30 min incubation at 37 °C under gentle shaking, cells were separated from residual emulsion by centrifugation at 250 × g for 5 min at 4 °C and washed twice with Hank’s buffer. Finally, the cells were suspended in modified relaxation buffer (200 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 10 mM PIPES-HCl, pH 6.8), treated with 5 mM MgCl2, 1 mM 1,4-dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.25 mg/ml soybean trypsin inhibitor and subjected to nitrogen cavitation (200 p.s.i. for 20 min). After addition of 1% Na2EDTA, the suspension was centrifuged at 48,000 × g for 30 min. Liberated phagocytic vesicles floating on the top of the tubes were collected with a spatula and resuspended in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4. Inside-out plasma membrane vesicles were frozen in liquid nitrogen and stored at −20 °C until use. Protein was determined using the bicinchoninic acid assay (21).

Electron Microscopy of Isolated Vesicles—Vesicles in modified relaxation buffer were added to the columns in a volume of 1–3 ml and incubated for 10 min. Nonadherent vesicles were eluted with ice-cold modified relaxation buffer, and protein in the fractions was quantified using the bicinchoninic acid assay (21). About 95–98% of the phagocytic vesicles were found in the first 6 ml of the eluate, whereas intact PMN almost completely (>95%) adhered to the columns. Based on the assumption that ConA receptors are found exclusively on the extracellular surface of the plasma membrane of PMN (22), from these experiments a low contamination of the inside-out membrane vesicles by right side-out vesicles or plasma membrane fragments was concluded.

Electron Microscopy of Isolated Vesicles—Vesicles in modified relaxation buffer were fixed in 3% glutaraldehyde at 4 °C overnight. Material was rinsed with relaxation buffer, followed by a 1.5-h fixation with osmium tetroxide in the same buffer. For en bloc staining, vesicles were incubated with 1% uranyl acetate in 20% acetone for 1.5 h, and dehydration was performed with a graded acetone series. Samples were then infiltrated and embedded in Spurr’s low viscosity resin. After polymerization, sections were cut, mounted on collodion-coated copper grids, and post-stained with aqueous lead citrate (3%, pH 13.0). All micrographs were taken on an EM 109 electron microscope (Zeiss, Oberkochen, Germany).

Arachidonic Acid Uptake into Inside-out Plasma Membrane Vesicles—The standard incubation medium contained plasma membrane vesicles (20 μg of protein) in modified relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 10 mM PIPES-HCl, pH 6.8) in a final volume of 200 μl. Tritiated arachidonic acid was added as a 50% ethanolic solution. The final ethanol concentrations never exceeded 0.5% and did not influence transport activity. Since a substantial portion of the added arachidonic acid was bound to the walls of the container, the actual arachidonate concentration in each experiment was determined by scintillation counting of aliquots of the incubations. All concentrations of arachidonate were corrected for adsorption losses to container walls. The incubations were carried out at 37 °C and terminated by the addition of 800 μl of ice-cold stop solution containing modified relaxation buffer, pH 6.8, 0.5 mM pentachlorphenol, and 0.5% albumin and then further incubated for 5 min on ice. The sample was pipetted onto a Schleicher & Schuell filter GF 52 (24 mm) and filtered using a filtration manifold (DN 025/1, Schleicher & Schuell, Dassel, Germany). Subsequently, vesicles were washed with 1 ml of stop solution and 3 ml of relaxation buffer. The filters were placed in scintillation vials and left overnight in 5 ml of Rotizint Eco Plus (Buch, Karlsruhe, Germany) before radioactivity was counted and corrected for adsorption of unbound arachidonate to the filters.

RESULTS

Vesicle Preparation—Vesicles prepared for electron microscopy consisted primarily of membrane-bounded vesicles (0.1–1.2 μm) and some associated granules. These isolated vesicles were identical to the phagocytic vesicles seen in intact cells after phagocytosis of albumin-coated mineral oil droplets. The vesicle membrane was clearly trilaminar and identical in appearance and dimensions to the plasma membrane. The membrane encloses the lipid droplet with an amorphous electron-dense periphery representing the albumin coat of the lipid droplet. There was essentially no contamination of the vesicles by mitochondria, glycogen particles, nuclei, or other cytoplasmic matter except for some granules associated with the vesicles, Fig. 1. Application of phagosomes to a ConA affinity column revealed that only a small fraction (~2–5%) of the vesicles was bound to the column, whereas intact PMN adhered to the affinity material (>95%), demonstrating that the vesicles had indeed the inside-out orientation with little contamination of plasma membranes with exposed ConA receptors.

Vesicle Association of Arachidonic Acid—Various concentrations of [1H]arachidonic acid were added as an ethanolic solution to a vesicle suspension (20 μg of protein in modified 200 μl of modified relaxation buffer) and incubated at 37 °C. A significant and constant portion of the added [1H]arachidonic acid was bound to the walls of the incubation container. About 50% of the added [1H]arachidonic acid was recovered when the incubation mixture from the Eppendorf tube was transferred to a scintillation vial and counted for radioactivity. From the recorded association in the buffer (50%), a constant arachidonic acid portion (70.1%) associated with the vesicles in the concentration range tested (0.24–9772 pmol/sample) and was recovered with the vesicle membranes after rapid filtration (Fig. 2). During a 30-min incubation, the vesicle-associated arachidonate concentrations did not change.

Adsorption of arachidonate to container walls is a common observation (23), which makes it necessary to correct arachido-
Arachidonate concentrations for adsorption losses in every experiment. Vesicle association of unbound arachidonate is thought to represent absorption to the plasma membrane (24) and uptake into the intravesicular space. To measure uptake accurately and to distinguish internalized ligand from that merely bound to the external surface of the vesicles, it is essential to employ a wash solution that blocks influx and efflux after certain periods of incubation and removes ligand bound to the external membrane of the vesicles (25).

In previous studies, washes with a 0.1–0.5% albumin solution with or without addition of 200 μM of the transport inhibitor phloretin were generally used to remove fatty acids, which are exchangeably bound to the outer membrane leaflet, while preventing efflux (25–29). We employed a 0.4% albumin solution containing 0.4 mM pentachlorophenol (PCP) for this purpose since PCP is a stronger influx/efflux inhibitor in our system.

To evaluate the effectiveness of this wash solution in incubations with vesicle preparations, vesicle aliquots (20 μg of membrane protein) were incubated at 37 °C with various amounts of [3H]arachidonic acid at different time points, and vesicle association of radioactivity was measured after treatment with ice-cold BSA/PCP (stop/chase solution) as described under “Materials and Methods.” The vesicle-associated arachidonate resistance toward BSA/PCP extraction increased linearly within the first 120 s of incubation at 37 °C. The linear regression line of this time course was extrapolated to zero time to give the amount of arachidonate that could not be extracted by the BSA/PCP wash (determined for every arachidonate concentration tested). As much as 98.3% of the merely bound arachidonate at zero time could be extracted from the vesicles with BSA/PCP stop solution. A similar result was obtained when vesicles were incubated at 0 °C instead of 37 °C, even after prolonged incubation times.

The non-extractable arachidonate increased in a linear fashion with increasing amounts of arachidonate (0.24–9772 pmol), Fig. 2. Thus, under the conditions employed, no saturable binding to putative receptors became apparent in the concentration range (1.22 nM to 48.9 μM) tested.

Arachidonate Export in Pig Neutrophils

**FIG. 2.** Binding of arachidonate to vesicle membranes. Inside-out plasma membrane vesicles (20 μg of protein in 200 μl of modified relaxation buffer) were incubated with [3H]arachidonate (0.24–9772 pmol, corrected for adsorption losses to the container) at 37 °C and treated with 800 μl of ice-cold relaxation buffer with (▲) or without (●) addition of 0.4% BSA, 0.4 mM PCP (●) for 5 min. Vesicle-associated radioactivity was determined after rapid filtration and scintillation counting of the filters.

**FIG. 3.** Time course of arachidonate uptake into phagosomes. Vesicles (20 μg of protein) were incubated with 4 pmol of [3H]arachidonate in 200 μl of modified relaxation buffer, pH 6.8 at 37 °C. Reactions were terminated by transferring an aliquot into 4 volumes of ice-cold stop/chase solution containing 0.5% BSA and 0.5 mM PCP. After 5 min on ice, incubations were filtered, and vesicle-associated radioactivity was determined by scintillation counting of the filters. An aliquot of the incubation at 150 min (arrow) was treated with stop/chase solution in a similar way but was sonicated three times for 10 s during the extraction period before recovery of membrane (vesicle-associated) radioactivity was determined (mean ± S.D., n = 6).

**Arachidonic Acid Influx into Inside-out Membrane Vesicles—** Vesicles were incubated with 20 nM [3H]arachidonate (4 pmol/200 μl) in modified relaxation buffer, pH 6.8 at 37 °C, and reactions were terminated after various time points by treatment with ice-cold BSA/PCP stop solution as described under “Materials and Methods.” During incubation, the non-extractable portion of vesicle-associated arachidonate increased from 68 fmol (1.7%) at zero time to approximately 1 pmol (58.8%) after 60 min (total arachidonate corrected for losses to the test tube represents 1.7 pmol = 100%). Fig. 3. Increase of non-extractable arachidonate associated with the membrane vesicles could either reflect a time-dependent tight binding to high affinity binding sites at or in the membrane vesicles or influx to an as yet undefined compartment within the vesicles that is not accessible to BSA/PCP extraction. To distinguish between these possibilities, incubation of vesicles at 37 °C was terminated after 150 min by addition of ice-cold BSA/PCP stop solution and subjected to a 3 × 10-s sonication treatment on ice and filtered. Almost complete loss of vesicle-associated arachidonate that had been accumulated during incubation at 37 °C was observed after physical destruction of the vesicles by sonication, Fig. 3. To check whether the loss of radioactivity was due to the loss of sonicated membranes, aliquots of the same incubation mixture were subjected to sonication and filtration in the same way as before, except that the BSA/PCP solution was replaced by albumin-free modified relaxation buffer. These control experiments revealed that the recovery of vesicle membranes was not influenced by the sonication procedure, suggesting that the loss of radioactivity was due to liberation of arachidonic acid from the intravesicular space. Thus, the time-dependent vesicle association of non-extractable arachidonate is consistent with influx but not with binding to high affinity binding sites on the vesicle membranes.

**Inhibition of Arachidonate Influx and Efflux by Pentachlorophenol—** Vesicles (20 μg of protein/200 μl) were incubated with 4 pmol of arachidonic acid at 37 °C, and internalized [3H]arachidonic acid was quantitated after BSA/PCP treatment after various time points by rapid filtration and scintillation counting. Uptake was linear over a period of 120 s. To measure influx inhibition by PCP, PCP was added (0.4 mM final concentration)
of the incubation mixtures received an addition of 0.4 mM PCP, and inhibitors were preincubated with vesicles in modified relaxation buffer for 15 min at 37 °C. Reactions were started by addition of 20 nM arachidonate, and internalized arachidonate was measured as described in the legend for Fig. 3 over the first 120 s (five time points). Initial influx rates were calculated from the slope of the linear regression line from experiments performed in triplicate (mean ± S.D.). AA, arachidonic acid; DISA, 3,5-diiodosalicylic acid; NEM, N-ethylmaleimide.

**Materials and Methods.**

Vesicles, 20 µg of protein in 200 µl of modified relaxation buffer, were incubated with 4 pmol of [3H]arachidonic acid at 37 °C. Vesicle-associated arachidonate after various time points was determined in the incubation mixtures (with or without additions) as described under "Materials and Methods." A, at time points indicated (arrow), aliquots of the incubation mixtures received an addition of 0.4 mM PCP, and incubation at 37 °C was continued (▲, □). An aliquot was incubated with no addition of PCP to monitor influx without inhibitor (●, ■). B, at time point indicated (arrow), half of the incubation mixture received either 0.4% BSA, 0.4 mM PCP (▼) or 0.4% BSA (▲), and incubation was continued at 37 °C before internalized arachidonate was determined.

**Fig. 4. Inhibition of arachidonate influx and efflux by PCP.** Vesicles, 20 µg of protein in 200 µl of modified relaxation buffer, were incubated with 4 pmol of [3H]arachidonic acid at 37 °C. Vesicle-associated arachidonate after various time points was determined in the incubation mixtures (with or without additions) as described under “Materials and Methods.” A, at time points indicated (arrow), aliquots of the incubation mixtures received an addition of 0.4 mM PCP, and incubation at 37 °C was continued (▲, □). An aliquot was incubated with no addition of PCP to monitor influx without inhibitor (●, ■). B, at time point indicated (arrow), half of the incubation mixture received either 0.4% BSA, 0.4 mM PCP (▼) or 0.4% BSA (▲), and incubation was continued at 37 °C before internalized arachidonate was determined.

to aliquots of the suspension after 0 or 75 s. Addition of 0.4 mM PCP prevented influx of arachidonate. Furthermore, internalized arachidonate concentrations at the time of PCP addition remained almost at the same level over the entire incubation period of 1000 s, demonstrating an efficient and long lasting influx inhibition effect of PCP, Fig. 4.

Since PCP did not block influx into vesicles over prolonged incubation times at 37 °C, but also prevented losses of internalized arachidonic acid during the incubation, we tested the hypothesis whether PCP could also prevent vesicle efflux. Incubation of vesicles (20 µg of protein/200 µl) with 4 pmol of arachidonic acid resulted in a time-dependent increase of internalized arachidonate. Addition of BSA (0.4% final concentration) stopped the accumulation of arachidonate in the vesicles, and a time-dependent loss of intravesicular arachidonate was observed, reaching close to zero time levels after 50 min. In contrast, addition of BSA/PCP (0.4 mM PCP, 0.4% BSA) only resulted in a very little loss of internalized arachidonate.

Inhibition of vesicle association as well as vesicle dissociation of the non-BSA/PCP extractable arachidonate cannot be explained on the basis of high affinity binding to putative receptors. Furthermore, inhibition of influx and efflux of arachidonate by the union transport inhibitor PCP suggested a non-diffusional uptake and release of arachidonate.

**Effect of Anion Transport Inhibitors on Arachidonate Influx**—To study the effect of common anion transport inhibitors on arachidonate transport, a variety of structurally different inhibitors were preincubated with vesicles in modified relaxation buffer for 15 min before 4 pmol/200 µl of [3H]arachidonic acid were added. To determine internalized arachidonate after various incubation periods, 200-µl aliquots of the suspension (20 µg protein) were pipetted at desired time points into 0.8 ml of cold 0.5% BSA, 0.5 mM PCP and filtered after 5 min extraction time. Incubations were performed in triplicate, and initial influx rates were computed from the slope of the linear regression line over the first 120 s after arachidonate addition. Strong inhibition of the initial influx was observed by 1 mM quercetin (11.5 ± 1.5% of control, mean ± S.D.), 0.5 mM phloretin (9 ± 5.6%), 0.5 mM doidosalicylic acid (10.3 ± 6.8%), and 0.5 mM pentachlorophenol (1.3 ± 0%), respectively (Fig. 5). N-Ethylmaleimide had little, if any, effect on arachidonate influx. However, three other compounds that inhibit monocarboxylate (18) or prostaglandin transport (30), 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid, α-cyano-4-hydroxycinnamate, and the dye bromcresol green, showed no effect on arachidonate influx. The effectiveness of common anion transport inhibitors is suggestive of non-diffusive uptake of arachidonic acid into the vesicles.

**Evidence for a Protein-facilitated Transport of Arachidonate**—Proteolytic digestion of vesicles is often employed as a key experiment to decide whether or not proteins are involved in transport processes. Since proteins facilitating transmembrane movement of ligands are often partially exposed on the membrane surface, they can be digested by proteases without damaging the membrane barrier (31). Vesicles were preincubated in modified relaxation buffer with or without addition of Pronase (1 mg/ml) or trypsin (2.5 mg/ml) overnight. Preincubation with either Pronase or trypsin at 37 °C strongly inhibited the initial arachidonate influx into the vesicles. Pronase and trypsin digestion reduced initial influx rates to 12.7 ± 4.6 and 5.6 ± 2.1% of control (mean ± S.D., n = 3), respectively. Preincubation at 0 °C had no effect on the influx rates, suggesting that influx inhibition is indeed mediated by the enzymatic properties of the proteases and not by unspecific protein binding as seen with albumin.

**Kinetics of Arachidonate Influx**—Inhibition of arachidonate influx by proteases suggested that at least one step in the mechanism of arachidonate influx is protein-dependent. Thus, arachidonic acid influx should be saturable by arachidonate. Initial arachidonic acid influx rates were determined as described above after addition of various amounts of arachidonate.
The slope of the Arrhenius plot an activation energy of 11.6 S.lope
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ucleus at a protein concentration of 100 m
and an optimum transport rate was measured at 51 °C (7.2
 influx was almost completely abrogated (0.22 pmol/min
strated a marked temperature dependence, Fig. 7. At 0 °C
first 120 s as described above. Arachidonate influx demon-
ning to Lineweaver-Burk giving a linear regression line (Fig. 6,
3
m
(3.0 ± 0.2 nmol/min × mg) was obtained (mean ± S.D., n = 3–6). The data were replotted according to Lineweaver-Burk giving a straight line with no apparent breaks in the curve (inset). AA, arachi-
donic acid.

(Final concentration 1.2 nM to 86.5 pm, corrected for adsorption
losses to the container). At low ligand concentrations, the ini-
tial influx rates increased in a linear fashion with increasing
arachidonic acid concentrations and appeared to saturate at
higher arachidonic acid concentration (Fig. 6). The data were fit-
ted against the Michaelis-Menten equation by nonlinear regres-
sion analysis. At a vesicle protein concentration of 100 µg/ml, a half-maximal in-
flux rate was calculated for an arachi-
donate concentration of 5.7 ± 1.9 µM, and a V_m of 3.0 ± 0.2
nmol/min × mg was obtained. The data were replotted accord-
ing to Lineweaver-Burk giving a linear regression line (Fig. 6, in-
set).

Temperature Dependence of Arachidonate Influx—To deter-
mine whether arachidonate influx is a temperature-dependent
process, vesicles were incubated at temperatures between 0
and 71 °C, and initial influx rates were determined over the
first 120 s as described above. Arachidonate influx demon-
strated a marked temperature dependence, Fig. 7. At 0 °C
influx was almost completely abrogated (0.22 pmol/min × mg),
and an optimum transport rate was measured at 51 °C (7.2
pmol/min × mg), Fig. 7, inset. The temperature dependence of
the initial influx rates (0–47 °C) was tested according to Ar-
rhenius where ln V_max is plotted against 1/T as shown in Fig. 7,
inset. A linear relationship was found (r = −0.9890), and from
the slope of the Arrhenius plot an activation energy of 11.6 ±
0.3 kcal/mol (mean ± S.D., n = 3) could be calculated.

Essentially no influx could be observed at 71 °C (0 pmol/
min × mg) and transport competence could not be restored by
lowering the temperature back to 37 °C. Inactivation of trans-
port function might be due to denaturation of a putative
arachidonate carrier protein or physical damage of the vesicles. Electron micro-
graphic pictures taken from the vesicles before and after a
10-min incubation at 70 °C showed that the vesicles were in-
deed heavily damaged by heat treatment. In fact, it was not
possible to find any intact vesicle in representative pictures of
more than 100 vesicles (data not shown).

Effect of pH on Arachidonic Acid Influx—To study the effect
of pH on initial arachidonic acid influx, the extravesicular pH
was varied from pH 9.8 to 3. At an alkaline pH higher than 8

(Fig. 6. Saturable uptake of arachidonate into phagosomes. Various concentrations of [3H]arachidonate were incubated with vesicles at a protein concentration of 100 µg/ml. Initial influx rates were determined as described in the legend to Fig. 5 and plotted against the arachidonate concentration (corrected for adsorption losses to the container walls). For the calculation of the kinetic parameters, data were fitted against the Michaelis-Menten equation by nonlinear regression analysis based on the least-squares method (line). A half-maximal influx rate was observed at an arachidonate concentration of 5.7 ± 1.9 µM, and a V_max of 3.0 ± 0.2 nmol/min × mg was obtained (mean ± S.D., n = 3–6). The data were replotted according to Lineweaver-Burk giving a straight line with no apparent breaks in the curve (inset). AA, arachidonate acid.}

(Fig. 7. Temperature dependence of arachidonate influx. Temperature dependence (0–71 °C) of arachidonate influx into phagosomes. Initial influx rates were determined as described in the legend for Fig. 5. The data (temperature range 0–47 °C) was replotted according to Arrhenius (inset). Graph indicates strictly linear relationship with no apparent breaks in the curve. Slope signifies activation energy of 11.6 ± 0.3 kcal/mol. Results are from three experiments. AA, arachidonate acid. Almost no influx could be measured (0.1 pmol/mg × min at pH 9.8) and was indistinguishable from similar incubations where PCP had been added. Lowering the pH to 6.0 increased the initial influx (12.7 pmol/mg × min) which could completely be inhibited by PCP, Fig. 8. At pH values below 6, initial influx is again suppressed, and influx at pH 3 is indistinguishable from that observed in the presence of PCP (0.45 pmol/mg × min). The enhancement of arachidonate influx by acidification and suppression by alkalinization in the pH range from 6 to 9 is compatible with an H+ + arachidonate cotransport system but may also be due to allosteric effects of pH on the hypothetical carrier protein. Electron microscopic examination of vesicle integrity at low pH values revealed that vesicles preincubated at pH 4.5 were almost indistinguishable from those incubated at neutral pH, whereas at pH 3.5 about 20% of the vesicles appeared to be damaged. Thus, decrease of transport activity at low pH values appears not to be predominantly due to vesicle destruction. Notably, PCP inhibited influx almost completely over the whole pH range tested, further supporting the hypoth-
eses that arachidonic acid influx is non-diffusive in nature even at
pH values below 6 where arachidonic acid should be in a
non-ionic state. A pH-induced change in the binding of arachi-
donate to putative receptors appears unlikely since sonication
of vesicles just prior to filtration liberates almost all of the
arachidonate (Fig. 2) without affecting membrane recovery.

Effect of Extravesicular ATP, Na+ and K+ Concentration on Arachidonic Acid Influx—We next investigated the influence of extravesicular Na+ and K+ concentrations. The relaxation buffer was replaced in different experiments by buffers (10 mM HEPES-KOH, pH 6.8) containing 0–200 mM NaCl or buffers (10 mM HEPES-NaOH, pH 6.8) containing 0–200 mM KCl. In a series of experiments, modified relaxation buffer containing various ATP concentrations (0.5–10 mM) or an ATP-regenerating
system was used as external medium. All these replace-
ments had no impact on the initial arachidonate influx or efflux rates (data not shown).

DISCUSSION

Fatty acid trafficking through membranes is very often thought to be purely by passive diffusion driven by fatty acid gradients (16). In contrast, our findings on arachidonate export support the view (15, 28, 31) that transmembrane fatty acid movement is a protein-dependent and saturable process that
Thus in macrophages, most of the arachidonate released exists in pools of arachidonate generated by different lipases (38, 39) and macrophages support evidence for the possibility of different pools of arachidonate generated by different lipases (38, 39). Thus in macrophages, most of the arachidonate released into the medium is derived from a secreted type II PLA₂ (sPLA₂) acting on the outer surface of the cell, while a group IV PLA₂ (cPLA₂) acting intracellularly is coupled to eicosanoid biosynthesis and is only responsible for a portion of the arachidonate released into the medium. In PMN, the situation may be similar, although direct evidence for secretion of sPLA₂ activity has been elusive (40). Alternatively, activated neutrophils may also generate arachidonate by the concerted action of phospholipase C or D (41) and a lipase located at the inner leaflet of the plasma membrane.

Regardless of the source, unesterified arachidonate dissolved in plasma membranes has been shown to influence a variety of membrane properties in different tissues including K⁺ channels (42, 43), Cl⁻ channels (44), proton pumping (45), Ca²⁺ permeability (46), and membrane ATPase (47). Therefore, it may be important that unesterified arachidonate in the inner leaflet of the plasma membrane is removed by an efficient export mechanism to clear the membrane from arachidonic acid to prevent disturbance of normal membrane function. Thus, export might be an additional mechanism apart from the rapid reacylation in the cytosol to keep the arachidonate level well below 3 pmol/million PMN (48) and may contribute to the release of arachidonate from PMN during cell activation.

Differential sensitivity toward anion transport inhibitors (18, 30, 49) makes it possible to discriminate between different transport pathways. So far, three different Cl⁻ channels and four different anion transport activities have been identified in neutrophils as follows: a Cl⁻/HCO₃⁻ exchanger (50), a sulfate carrier (51), a H⁺ + lactate-carrier (52), and a H⁺ + lipoxin A₄ cotransporter (49). Lactic acid fluxes are completely blocked by N-ethylmaleimide, and the sulfate carrier is very sensitive to 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid, whereas arachidonate export is sensitive to neither of these inhibitors at roughly comparable concentrations. The Cl⁻/HCO₃⁻ exchanger displays a pH dependence opposite to that for arachidonate transport. In contrast, many of the kinetic characteristics and the inhibitor profile for arachidonate export are shared by the H⁺ + LXA₄ cotransporter that imports the eicosanoid LXA₄ into PMN. Similar to LXA₄ uptake into PMN, arachidonate influx into phagosomes displays a striking pH dependence with almost no transport activity at pH 8 and highest activity at pH 6. ATP, Na⁺, or K⁺ dependence was not noted in either case. With respect to the inhibitors, both transporters are sensitive toward PCP, 3,5-diodosalicylic acid, but not to N-ethylmaleimide, iodoacetate, and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid. The energy of activation for both transporters was relatively low, 15.0 kcal/mol for the trihydroxylated eicosanoid LXA₄ and 11.6 kcal/mol for arachidonate. Substrate saturation was not seen with the LXA₄ transporter but was evident in the case of arachidonate transport at relatively high concentrations. It has not yet been clarified if arachidonate influx and efflux in PMN is mediated by the same protein, facilitating the electroneutral diffusion of arachidonate + H⁺ species through the membrane similar to the lactate + H⁺ cotransporter in PMN (52) and erythrocytes (18). Alternatively, two independent transport systems may exist. The question of which other eicosanoids and fatty acids are accepted by the arachidonate transporter has not been addressed in this investigation and remains to be elucidated in the future.

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FIG. 8. Effect of pH on arachidonate uptake into vesicles. Initial influx rates were determined as described in Fig. 5 in the absence or presence of 0.4 mM PCP in medium wherein pH, was varied between 3 and 9.8. Optimum transport activity was observed at an extravesicular pH of 6.
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