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INTRACELLULAR PHOSPHOLIPASES A

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Contents

I. Introduction

The existence of enzymes catalyzing the release of fatty acids from phospholipids was proposed over a hundred years ago, when it was observed that incubation of phosphatidylcholine with pancreatic juice resulted in formation of free fatty acids (see Ref. 1 for

Abbreviations: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

historical review). The subsequent finding in 1903, that cobra venom converted phosphatidylcholine into a product which possessed the property of being able to hemolyze red blood cells, directed much of the early research in this area towards the phospholipid-hydrolyzing enzymes in snake venoms and towards the elucidation of the structure of the lytic product. Around 1915 it was established that this was a phosphatidylcholine from which one fatty acid had been removed. For obvious reasons, the term lysophosphatidylcholine was introduced for this product. It was not until about 1960, however, that the problem as to which fatty acid was actually removed by the snake venom phospholipase A was definitely settled as being the one esterified to the *sn*-2-position [2,3]. Such enzymes are found in all snake venoms and in the poisons of bees, wasps and scorpions [4].

Indirect evidence for the occurrence of phospholipase A in animal tissues other than pancreas was obtained by Francioli [5] as early as 1934, when appreciable quantities of lysophosphatidylcholine were found in dried, but not in fresh or autoclaved, preparations of heart, liver, spleen, brain, muscle, thymus and prostate. With the availability of isotopically labelled substrates and the known positional specificity of the snake venom phospholipases A it became clear in the mid-sixties that several mammalian tissues contained lipolytic activities attacking the fatty acyl ester bonds at either position of the glycerol moiety [6,7]. Thus, intracellular phospholipases A were designated as A_1 and A_2 activities, producing 2-acyl and 1-acyl lysophospholipids, respectively. The extracellular phospholipases A from snake venoms as well as the enzymes secreted by pancreas all belong to the A_2 type [8]. The latter group of enzymes, although studied in much more detail from both a mechanistic and structural point of view [9–12], is not reviewed here. This review deals with the vast field of intracellular phospholipases A and their involvement in membrane processes. Many proposals for the function of intracellular phospholipases A have been put forward in the literature and I shall try to evaluate the available evidence. Of course, the question as to the possible structural relationship between intracellular phospholipases and the well documented venom and pancreas phospholipase A_2 will be considered. Also, some comparative aspects of intracellular lipases and lysophospholipases will be included. While some examples of the widespread occurrence of intracellular phospholipases A will be given, it is not the purpose of this review to give an extensive treatise on this subject. Reasonably up-to-date enumerations can be found in various reviews [8,13–15]. Assay methods, with special reference to determine the relatively low activities generally associated with crude preparations of intracellular phospholipases, were recently discussed [16].

Phospholipases A activities have been found in almost every cell where they have been sought. Thus, such activities have been described in bacteria [17], amoeba [18], insects [19], plants [20] and many mammalian tissues including liver [21], spleen [22], lung [23], brain [24], heart [25], retina [26], adrenal medulla [27], intestinal [28] and stomach mucosa [29] as well as in erythrocytes [30], platelets [31], polymorphonuclear leukocytes [32] and alveolar macrophages [33]. Not only do many of these cells contain both phospholipase A_1 and A_2 activities, but in addition their intracellular location is not restricted to a single subcellular site. Although there may be considerable variation, especially between eukaryotic cells, in the distribution of phospholipases A, the ubiquity of these enzymes can be adequately illustrated by considering rat liver tissue.

II. Rat liver phospholipases A

Without going into details or discussing in full length the controversial results that have sometimes been obtained, it seems justified to summarize the phospholipase distribution in the rat hepatocyte as indicated in Table I.

Plasma membranes contain both phospholipase A_1 [34,35] and phospholipase A_2 [36, 38]. Both enzymes are optimally active at an alkaline pH of about 9 and require Ca^{2+} for optimal activity. The ratio in which the two enzymes express themselves in vitro appears to vary considerably depending on the substrate used and the method of isolation of the plasma membranes [38,39]. Since pancreatic lipase has been shown to exhibit phospholipase A_1 activity [40], Newkirk and Waite [34] investigated whether the plasma membrane phospholipase A_1 activity could be ascribed to a lipase. They concluded that this was not the case as no hydrolysis of radioactively labeled tripalmitoylglycerol was observed. However, Colbeau et al. [41] reinvestigated the problem using triolein emulsified in gum arabic as substrate and found both a triglyceride and a monoglyceride lipase in plasma membranes. Both enzymes were optimally active at pH 9. The differential effects observed during heat treatment and solubilization by salts and detergents tended to suggest that the triglyceride lipase, the monoglyceride lipase and the phospholipase A_1 were different protein entities. In general, such studies are subject to many pitfalls. For example, it is difficult to rule out that the activity of a single, membrane-associated enzyme with broad substrate specificity is influenced to different extents by heat treatment, the presence of detergents or (partial) solubilization depending on the substrate used (see subsection IIIA). The phospholipase A_1 activity could easily be solubilized from isolated plasma membranes by heparin treatment [42]. Evidence was provided to show that this enzyme is most likely identical to the phospholipase A_1 activity purified from post-heparin plasma [43]. This enzyme catalyzes both hydrolytic and acyl-transfer reactions with a variety of substrates and it has been suggested that the enzyme should be more appropriately named monoglyceride acyltransferase, since monoglyceride is the preferred substrate and transacylation is the predominant reaction [44]. These findings make it very likely that the enzyme is identical, after all, to the monoglyceride lipase in plasma membranes, despite the differences observed by Colbeau et al. [41] and illustrate the pitfalls discussed above. The following discrepancy is probably a further example thereof. According to Waite and Sisson [44], the heparin-solubilized enzyme does not hydrolyze

TABLE I

PHOSPHOLIPASE A DISTRIBUTION IN RAT LIVER

+, stimulated; –, inhibited; o, no effect; n.i., not investigated.

Subcellular site	Main activity	pH optimum	Ca^{2+} effect	Reference
Plasma membrane	$A_1 + A_2$	8.0–9.5	+	34–39
Microsomes	A_1	8.0–9.5	+	21,35,37,47
Golgi membrane	$A_1 + A_2$	8.0	+	37
Mitochondria	A_2	8.0–9.0	+	35,50–55
Lysosomes	$A_1 + A_2$	4.0–5.0	–	35,58,59
Cytosol	$A_1 + A_2$	7.4	n.i.	21
	A_1	3.6	o	60,61

triacylglycerol. Inasmuch as this enzyme is thought to be identical to the post-heparin plasma phospholipase A_1 [43], it is relevant to note that Ehnholm et al. [45] have isolated a heparin-released phospholipase A_1 from human plasma with almost equal hydrolytic activity against triolein and phosphatidylethanolamine. Some evidence has been obtained which suggests that a large part of the phospholipase A_2 of rat liver plasma membranes is present in an inactive form which can be converted into active enzyme under certain conditions [39] (see subsection IVA). Only when present in its active form could the enzyme be released from the membrane by high salt concentrations. The membrane-bound enzyme showed normal Michaëlis-Menten kinetics with sonicated phosphatidylethanolamine as substrate. In contrast, the solubilized phospholipase A_2 showed irregular kinetics with a marked increase in activity above 35 μM substrate concentration [46]. A similar V/S curve was noticed for the phospholipase A_1 in plasma membranes. Critical micelle concentration (CMC) determination in the assay medium by fluorescence measurements yielded CMC values in the range of 15–30 μM. The sharp increase in activity above 35 μM substrate was attributed to formation of lipid aggregates, and resembles the striking rate enhancement found for pancreatic phospholipases A_2 when assayed with substrates above the CMC [10]. It should be noted, however, that the measured CMC values are rather high and even several-fold greater than others have reported for lysophospholipids [280,313]. The kinetic behavior of the enzymes and other data such as pH dependency and preference for endogenous or exogenous substrate led the authors to hypothesize a different localization of phospholipase A_1 and A_2 in the plasma membrane [46]. Thus, phospholipase A_1 activity would be caused by a peripheral membrane protein with exposure of its active site to the external medium and would be most active with exogenous substrates. In contrast, phospholipase A_2 would be much more embedded in the membrane, would require the penetration of exogenous substrate prior to hydrolysis and therefore show a preference for endogenous substrate. As pointed out by the authors, these suggestions of a different localization of active sites in the membrane should be considered tentatively until more direct evidence becomes available (cf. Fig. 1).

The microsomal fraction isolated from rat liver contains both phospholipase A_1 and A_2 activities, but invariably the A_1 activity was reported to be much more prominent [21,35,37,47]. Attempts to differentiate between the phospholipase A_1 and a microsomal triglyceride lipase have yielded some indications that distinct enzymes may be involved. No phospholipase A_1 inhibition was observed with *p*-chloromercuribenzoate

Fig. 1. Schematic model for the activation of membrane-bound phospholipase A_2. Left, inactive conformation; right, active conformation. As discussed in the text, two possible mechanisms for this activation can be put forward, i.e., proteolytic action converting a prophospholipase into an active enzyme or complex formation between an inactive enzyme and an as yet undefined platelet factor to yield active phospholipase A_2. The model depicts schematically the observations made during the activation of liver plasma membrane phospholipase A_2, namely a decrease in hydrophobic binding accompanying the conversion of inactive enzyme into active enzyme [39].

under conditions causing 30% inhibition of the microsomal lipase [21]. A subsequent investigation [41] demonstrated equal sensitivity to heat treatment and similar extractability by increasing salt concentrations for both enzymatic activities. While these observations are in line with the existence of a single enzyme, partial delipidation of the microsomes with either acetone or phospholipase C had different effects on triglyceride and phosphatidylethanolamine hydrolysis. Whether these differences are related to varying physico-chemical properties of the two substrates or point to the presence of distinct protein entities cannot be established with certainty at present.

The few studies that have been performed to measure the phospholipase A activities of highly purified Golgi membranes have indicated the presence of A_1 and A_2 activities, with phospholipase A_1 predominating [37]. The purified Golgi membranes lacked several lipid biosynthetic activities, i.e., cholinephosphotransferase, acyl-CoA : 1,2-diacyl-*sn*-glycerol acyltransferase and acyl-CoA : 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase [37, 48]. Inasmuch as the latter enzymes are located either exclusively or mainly in the endoplasmic reticulum [14], these results demonstrated that the phospholipases A_1 and A_2 are true constituents of Golgi membranes and are not the result of microsomal contamination as the similar positional specificities observed for the microsomal phospholipases might suggest. It should be noted, however, that the exclusive localization of cholinephosphotransferase in endoplasmic reticulum membrane has recently been questioned. Jelsema and Morré [49] concluded that, although nearly 90% of the total activity of cholinephosphotransferase was accounted for by endoplasmic reticulum, the specific activity of the enzyme in the Golgi apparatus was 40% of the endoplasmic reticulum. This high value could not be accounted for on the basis of the approx. 5–10% contamination of the Golgi fraction by endoplasmic reticulum. Inasmuch as the specific activities of the phospholipases A_1 and A_2 in the Golgi fraction were 50 and 130%, respectively, of the values found for endoplasmic reticulum [37], the conclusion that these enzymes are true constituents of the Golgi apparatus seems to be still valid.

Mitochondria contain a Ca^{2+}-dependent phospholipase A_2 with an alkaline pH optimum. The enzymic activity appears to be present in both inner and outer mitochondrial membranes [35,54], although it is not yet known whether the same protein entity is present in both membranes. The apparent K_m value for phosphatidylethanolamine was reported [35] to be several-fold smaller for the enzyme in the outer membrane (90 as against 400 μM), but this can also be explained by a different environment for the protein. The phospholipase has been partially purified from total rat liver mitochondria by Waite and Sisson [55]. The 160-fold purified preparation hydrolyzed phosphatidylethanolamine optimally at pH 9.5, but phosphatidylserine hydrolysis was more extensive at pH 7.4, thus, clearly indicating the effect of environmental factors. So far, the mitochondrial enzyme constitutes the only membrane-bound phospholipase A from rat liver which has been purified to a reasonable extent. Unfortunately, further purification was hampered by extreme instability resulting in complete loss of activity.

In contrast to the membrane-bound phospholipases referred to above, rat liver lysosomes were found to contain soluble phospholipase A activities. Initially, it was assumed from the lack of accumulation of lysophosphoglycerides that one enzyme catalyzed the complete deacylation of phosphoglycerides [56,57]. Stoffel and Trabert [58] were the first to suggest the presence of two phospholipases with different positional specificity on the basis of selective inhibition studies. These results were confirmed by Nachbaur et al. [35] and Franson et al. [59]. The latter authors partially separated phospholipase A_1 and A_2 by gel filtration of the lysosomal soluble fraction on Sephadex G-200. Remarkably,

both enzymes were found to be inhibited rather than stimulated by Ca^{2+}, although to different extents, with phospholipase A_2 activity being more sensitive. It is not unlikely that the phospholipase A_1 activity may be associated with acidic lysosomal lipases or esterases. Acidic triglyceride lipase was mainly recovered in the soluble fraction of lysosomes [41]. Incubation of this fraction at 60°C resulted in a more rapid loss of lipase activity when compared with phospholipase activity, but this should not be used as an indication of separate enzymes since the phospholipase A in these studies was not defined as A_1 or A_2 activity.

Soluble phospholipase activities have also been detected in the cytosol fraction obtained from rat liver homogenates [21]. Due to the presence of active lysophospholipase(s), the initial site of attack in the hydrolysis of phosphatidylethanolamine could not be ascertained Inhibition of the lysophospholipase(s) with deoxycholate led to the accumulation of both 1-acyl and 2-acyl lysophosphatidylethanolamine, indicating the presence of both phospholipase A_2 and A_1. These activities were measured at neutral pH values. It is not known at present whether these enzymes occur in the cytoplasm of intact cells or if their recovery in the 100 000 × *g* supernatant is due to solubilization of these enzymes from other subcellular structures during the homogenization and fractionation procedure. This question applies particularly to a recently described acidic phospholipase A_1, which was found in the cytosol of many mammalian cells, including rat liver [60,61]. This enzyme could not be detected in the absence of detergents, but was markedly activated in the presence of acidic phospholipids, lysophosphatidylcholine and some non-ionic detergents such as Triton X-100 and Tween 20. Under these conditions, the enzyme exhibited highest activity at pH 3.6, thus resembling the lysosomal soluble phospholipase A_1. Based on several criteria, the authors believe, however, that the cytosolic enzyme is different from the lysosomal one.

It is clear from the above discussion that many basic questions about the intracellular phospholipases have remained unresolved. Is there any structural relationship between the phospholipases A_1 and the pancreatic and venom enzymes? Do the various subcellular membrane structures contain identical protein entities with either phospholipase A_2 or A_1 activity or does each subcellular structure possess different phospholipases? Likewise, the relationship, if any, between intracellular phospholipase A_1 and lipase activities remains largely unclear. Obviously, the answer to such questions must await purification of the enzymes and a careful comparison of the properties of these enzymes in a purified and reconstituted membrane-bound state. Considering that purification is a prerequisite to full characterization of enzymes, the vast field of intracellular phospholipases becomes suddenly very small. Nevertheless, several successful attempts to purify intracellular phospholipases have been undertaken in the last decade. The results thereof will be discussed next.

III. Purified phospholipases A

IIIA. Phospholipases A_1

Most of the intracellular phospholipases A that have been purified in earlier studies belonged to the class of A_1-type enzymes (Table II). Scandella and Kornberg [17], taking advantage of the marked stability of a membrane-bound phospholipase A of *Escherichia coli* B in SDS solutions saturated with butanol, purified this enzyme approx. 5000-fold to

TABLE II

PURIFIED PHOSPHOLIPASES A_1 AND PHOSPHOLIPASES B

PA_1, phospholipase A_1; PA_2, phospholipase A_2; LPase, lysophospholipase; MB, membrane-bound; S, soluble. +, required; –, not required.

Source	Main activity	Form	M_r	pH optimum	Ca^{2+} effect	Specific activity (units/mg)	Reference
E. coli B	PA_1 + *l* Pase	MB	29 000	8.4	+	2.0	17
E. coli K-12	PA_1 + PA_2 + LPase	MB	28 000	8.0	+	4.7	67
M. phlei	PA_1 + LPase	MB	45 000 *	8.0 **	–	0.7, 7.2	67
[illegible]	PA_1	S	26 000	5.5–6.5	–	1560	68
Pancreas	PA_1 + LPase	S	60 000	7.5	–	0.8	69
P. notatum	PA_2 + LPase	S	116 000	4.0	–	27	70,71
Brain	PA_1	S	75 000	4.2	–	0.04	72

* Another enzyme with M_r 27 000 showed similar substrate specificity.

** For neutral phospholipids; acidic phospholipids were optimally hydrolyzed at about pH 4.

near homogeneity. The purified enzyme was identified as a phospholipase A_1 by its product formation from synthetic mixed-acid substrates. The lack of triolein hydrolysis distinguished the enzyme from pancreatic and fungal lipases. Phosphatidylethanolamine and phosphatidylglycerol were hydrolyzed at the same rate, either in the presence or absence of Triton X-100. In contrast, diphosphatidylglycerol hydrolysis was stimulated about 100-fold by the addition of Triton and then proceeded at comparable rates as observed for the other major phospholipids of *E. coli*. 1-Acyl lysophosphatidylethanolamine was hydrolyzed twice as fast as phosphatidylethanolamine under the standard assay conditions, i.e., in the presence of Triton X-100. Apparently, 2-acyl lysophospholipids are not hydrolyzed as they represent one of the products obtained by action of the enzyme on diacyl phospholipids. On the basis of its substrate specificity, the enzyme can be denoted as a phospholipase A_1 with lysophospholipase activity.

Nishijima and coworkers [62] have isolated an enzyme from *E. coli* K-12 which most likely represents the K-12 analog of the *E. coli* B enzyme isolated by Scandella and Kornberg [17]. In both strains the enzyme appears to be located in the outer membrane [63–65] and is solubilized and partially purified by identical procedures [62]. However, there appears to be some difference in the positional specificity and sensitivity to SDS of the enzyme from the two sources. The *E. coli* B enzyme produced only 2-acyl lysophospholipids, while the K-12 enzyme formed both 1-acyl and 2-acyl lysophospholipids. This indicates that the initial attack in the deacylation of a diacyl phospholipid can take place at either the 2-position or the 1-position. The relative activity of the initial deacylation reactions could not be ascertained because the purified enzyme also hydrolyzed both positional isomers of lysophospholipid. The rate of deacylation of 1-acyl lysophosphatidylethanolamine was 5-times that of the 2-acyl isomer. As for the *E. coli* B enzyme, these reactions were carried out in the presence of 0.05% Triton X-100. The latter stimulated the hydrolysis of 1-acyl lysophosphatidylethanolamine somewhat, but inhibited by at least 80% the breakdown of 2-acyl lysophosphatidylethanolamine. Under the proper conditions, the K-12 enzyme, and probably also the *E. coli* B enzyme, should be able to catalyze a complete deacylation of diacyl phospholipids and thus can also be classified as phospholipases B according to the nomenclature rephrased by McMurray and Magee [66]. It follows from the above description of the substrate specificity that much of the apparent specificity depends on the detergent concentration. The action of this enzyme in the in situ situation will be discussed in subsection VD.

An enzyme with similar, but not identical, substrate specificity was purified from the membranes of *Mycobacterium phlei* [67]. Hydrolytic rates for 1-acyl and 2-acyl lysophosphatidylethanolamine were equal and about 2-fold higher than with phosphatidylethanolamine when tested at the same substrate concentrations. Yet, during incubation with phosphatidylethanolamine, small amounts of lysophosphatidylethanolamine accumulated which were shown to consist almost exclusively of the 2-acyl isomer. Thus, this enzyme can also be classified as a phospholipase B, but differs from the *E. coli* K-12 enzyme in the initial site of attack of diacyl phospholipids, which in this case is predominantly the ester linkage at the 1-position. It was stated that the rate of triolein hydrolysis was less than 1% of that for phosphatidylethanolamine, but it should be noted that values of 0.7 and 7.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for phosphatidylethanolamine hydrolysis are reported [67].

The most active and probably also the most specific phospholipase A_1 has been obtained from *Bacillus megaterium* spores [68]. In its action on phosphatidylglycerol the purified enzyme was stimulated about 25-fold by either the non-ionic detergent Triton

X-100 or anionic detergents such as taurocholate or deoxycholate. In the presence of Triton X-100, anionic phosphatidylglycerol was by far the best substrate and phosphatidylethanolamine was virtually inert. However, phosphatidylethanolamine hydrolysis was stimulated about 100-fold in the presence of taurocholate. Under these conditions, phosphatidylethanolamine hydrolysis amounted to 50% of that of phosphatidylglycerol. These data support the view that the enzyme prefers negatively charged substrate-detergent complexes. Tributyrin, even in the presence of taurocholate, was hydrolyzed at a rate less than 0.2% of that of phosphatidylcholine and phosphatidylethanolamine. Since the lysophospholipids produced were the 2-acyl isomers, the enzyme seems to be fairly specific for the 1-acyl ester linkage of phospholipids, i.e., it acts as a phospholipase A_1. It should be mentioned, however, that some activity was also noted with 1-acyl lysophosphatidylglycerol and that its substrate was only tested at low concentrations in the presence of detergents. The latter are known to inhibit most lysophospholipase activities. It cannot be ruled out, therefore, that the enzyme can catalyze the complete deacylation of diacyl phospholipids in the absence of detergents.

It is well known that detergents, by modifying the physico-chemical state of the substrate, exert a great influence on the rates of lipolytic reactions. The discussion in this section has provided several examples of this phenomenon. It should be realized, however, that when carried to its extreme this can completely change the apparent substrate specificity of an enzyme. We have demonstrated this for a protein isolated to homogeneity from bovine pancreas. In the absence of deoxycholate the enzyme was fully active on 1-acyl and 2-acyl lysophosphoglycerides with a 5- to 10-fold higher rate with the 1-acyl isomer [69]. Intact natural phospholipids were hydrolyzed at rates less than 1% of those observed for 1-acyl lysophospholipids. These rates were stimulated 25-fold by addition of optimal amounts of 1 mg per ml of deoxycholate. Under these conditions, equimolar amounts of fatty acid and 2-acyl lysophospholipid were produced, indicating that the enzyme acted as a phospholipase A_1. It turned out in subsequent studies that the deoxycholate concentrations used to obtain maximal stimulation of phospholipase A_1 activity caused complete inhibition of the lysophospholipase activity of this enzyme. In the presence of intermediate levels of detergent, both activities were expressed and the enzyme acted as a phospholipase B catalyzing the complete deacylation of phosphatidylcholine and phosphatidylethanolamine.

Similar observations were made for a phospholipase B purified to homogeneity from *Penicillium notatum* [70,71]. The ratio of lysophosphatidylcholine to phosphatidylcholine hydrolysis changed from 100 : 1 in the absence of Triton X-100 to about 1 : 1 in the presence of this detergent. Thus, only in the presence of Triton X-100 was some accumulation of lysophosphatidylcholine seen. This enabled the authors to determine the sequence of release of acyl groups during incubation with phosphatidylcholine. In contrast to the pancreatic enzyme, the *P. notatum* phospholipase B predominantly attacked the diacyl phospholipid initially at the 2-position.

An 80-fold purified phospholipase A_1, presumably of lysosomal origin, was obtained from acetone powders of human brain. The enzyme specifically released fatty acids from the 1-position of phosphatidylcholine and phosphatidylethanolamine in an assay medium containing Triton X-100 and taurocholate. Hydrolysis of lysophospholipids in the absence of detergents was not studied [72].

In summary, the enzymes described in this section have widely varying molecular weights and specific activities. Some require Ca^{2+} for their activity while others do not. Some occur intracellularly in membrane-bound form and others in soluble form. In most

cases, the lysophospholipase activity is at least comparable to and usually much greater than the phospholipase A activity, but this varies greatly with environmental factors such as the nature and concentration of detergent. Probably, most of the enzymes listed in Table II show phospholipase B activity under appropriate conditions. In this regard most enzymes attack the diacyl phospholipid initially at the *sn*-1-position, while others first attack the *sn*-2-position (*F. notatum*) or show less preference for either position (*E. coli* K-12). Virtually nothing is known about the factors determining the sequential release of acyl groups from the different positions in the diacyl phospholipid molecule. Little is also known about which substrates are attacked in vivo by these enzymes and the nature or fate of the products formed. Such information is difficult to deduce from the in vitro substrate specificity of these enzymes in view of the influences detergents exert thereon. In this respect, it will be interesting to see what action the enzymes have on membrane-embedded phospholipids and lysophospholipids and which intracellular factors, if any, mimic the in vitro function of detergents.

The lack of specificity for the *sn*-2-position and in most cases the lack of a clear-cut Ca^{2+} requirement as well as the molecular weights in SDS gels clearly distinguish these enzymes from the venom and pancreas phospholipases A_2.

IIIB. Phospholipases A_2

The purified phospholipases A_2 are listed in Table III. The rat spleen enzyme was obtained in soluble form from rat spleen homogenates by sonication, indicating that this is probably not a membrane-bound enzyme. The purified enzyme showed an isoelectric point of pH 7.4, a requirement for Ca^{2+} and specificity for the *sn*-2-position of phosphatidylethanolamine. Hydrolysis of phosphatidylcholine was inhibited by deoxycholate, whereas no significant inhibition was found with phosphatidylethanolamine as substrate. These properties are similar to those shown by pancreatic phospholipase A_2, except that phosphatidylcholine hydrolysis by the latter enzyme was stimulated by adding deoxycholate [74].

The presence of an active phospholipase A_2 in the insoluble pulmonary secretions of patients with alveolar proteinosis was demonstrated by Sahu and Lynn [75]. This enzyme is included in Table III, since it is one of the very few mammalian phospholipases A_2 that have been purified, to date, from sources other than pancreatic tissue. It is uncertain, however, whether this is an intracellular phospholipase and from which cell type it originates. Since airway cells or inflammatory cells are potential sources of this enzyme, the authors also studied phospholipase A_2 in isolated human polymorphonuclear leukocytes, rabbit alveolar macrophages and pig tracheal mucosa. The latter cell types contained anywhere from 5- to 10-times less phospholipase activity, on a mg protein basis, when assayed under optimal conditions for the pulmonary secretion enzyme. Starting from lyophilized powder, obtained after therapeutic bronchoalveolar lavage, the enzyme was solubilized by delipidation and purified to homogeneity [76]. The molecular weight as estimated by gel filtration and SDS-polyacrylamide gel electrophoresis amounted to 75 000 in both cases, strongly suggesting that the enzyme consisted of a single polypeptide chain. This was corroborated by the finding that only a single N-terminal residue, alanine, could be detected. It is interesting to note that the same N-terminus has been found in the pancreatic phospholipases A_2 [77]. No sugar residue could be detected in the phospholipase A_2 from pulmonary secretion. This property as well as the Ca^{2+} requirement and stimulation of phosphatidylcholine hydrolysis by deoxycholate is shared with the pancreatic enzymes.

TABLE III

PURIFIED PHOSPHOLIPASE A_2

+, required; S, soluble; MB, membrane-bound; U, unknown; PMN, polymorphonuclear.

Source	Form	M_r	pH optimum	Ca^{2+} effect	Specific activity (units/mg)	Reference
Rat spleen	S	15 000	7.0	+	5.0	73
Human pulmonary secretions *	U	75 000	8.0	+	1140	75,76
Rabbit exudate *	U	14 800	8.0	+	0.5	78
Rabbit PMN leukocytes	MB	14 000	7.5	+	5.0	317
Sheep erythrocytes	MB	12 000–18 500	8.0	+	4.7	79
Rabbit platelets	MB	12 000	9.0	+	0.8	85
Human platelets	MB	44 000	9.5	+	0.5	314

* (Intra) cellular source unknown.

Peritoneal exudates, produced in rabbits by injection of glycogen in saline, contained a soluble phospholipase A_2. A 300-fold purified preparation showed one band in normal and SDS-polyacrylamide gel electrophoresis. Gel filtration and SDS-gel electrophoresis both gave an estimated molecular weight of 14800. The enzyme required Ca^{2+} and showed a strict specificity for the *sn*-2-position [78]. The striking similarity in the enzymic and physical properties of the peritoneal fluid phospholipase A_2 and a membrane-associated phospholipase A_2 from polymorphonuclear leukocytes suggested that the soluble phospholipase in the exudate is probably of leukocyte origin.

A membrane-associated phospholipase A_2 from rabbit polymorphonuclear leukocytes was recently extracted from intact cells by treatment with 0.16 N H_2SO_4. The solubilized enzyme was purified over 8000-fold to yield a preparation with a specific activity of 5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ when assayed with autoclaved *E. coli* as substrate [317]. According to the authors, this specific activity is comparable to that of pure phospholipase A_2 from other sources in this assay system. The molecular weight of this phospholipase A_2 is about 14000.

Another clearly membrane-bound phospholipase A_2 which has been purified to near homogeneity was obtained from sheep erythrocytes by Kramer et al. [79]. Ghost membranes were extracted at low ionic strength to effect the release of weakly bound membrane proteins. Optimal solubilization of membrane proteins, including the phospholipase A_2, was achieved with buffer containing 0.5% SDS. This detergent inhibited the phospholipase A_2 almost completely and was replaced by cholate, which itself was unable to solubilize the enzyme from the membranes, in a subsequent gel-exclusion chromatography step using Sephadex G-75. The active fraction was further purified on an affinity adsorbent to yield a 2800-fold purified enzyme, which gave a single band on SDS-gel electrophoresis. The affinity adsorbent, initially developed by Rock and Snyder [80], was an alkyl ether analog of phosphatidylcholine coupled as ligand to AH-Sepharose 4B. The procedure is based on the fact that some, but not all, phospholipases A_2 only bind to their substrate in the presence of Ca^{2+}. Taking advantage of this property, the enzyme can be eluted from the affinity column with buffer containing EDTA. As demonstrated by Kramer et al. [79], this principle can still be applied in buffers containing 0.5% (w/v) cholate. A molecular weight of approx. 12000 was estimated for the sheep erythrocyte phospholipase A_2 from its behavior on Sephadex G-75 columns. Comparison with marker proteins in SDS gels yielded an apparent molecular weight of 18500. Studies on the substrate specificity of this enzyme demonstrated [81] a preferential release of polyunsaturated fatty acids, especially C22 fatty acids, compared to mono- and diunsaturated C18 acyl chains. This preference was observed equally well with phosphatidylcholine and phosphatidylethanolamine. Such a selective cleavage of acyl ester bonds in response to variation in chain length and unsaturation was not found with pancreatic phospholipase A_2. The sheep erythrocyte enzyme was about 10-times more active on phosphatidylcholine than on phosphatidylethanolamine and phosphatidylglycerol, while phosphatidylserine was not attacked at all. This preference for phospholipid polar groups is thought to be related to the lipid composition of ruminant erythrocyte membranes, which are remarkably low in phosphatidylcholine content. It is interesting to note in this respect that the phosphatidylcholine content of sheep erythrocytes increased up to 5-fold upon incubation with sheep [82] or human [83] serum provided its phospholipase was inhibited by EGTA or inactivated by pronase treatment. Inactivation of the phospholipase in intact erythrocytes by pronase or in resealed and leaky ghosts by chymotrypsin [84] led to the conclusion that the phospholipase is oriented towards the exterior of the

cell. Even after extensive degradation of membrane proteins, approx. 10% of the initial phospholipase activity remained. This may indicate that a part of the enzyme, including the active site, is embedded in the hydrophobic core of the lipid bilayer. In washed membranes the phospholipase was found to be inactive at Ca^{2+} concentrations below 10^{-5} M. A rapid increase in activity was observed between $5 \cdot 10^{-5}$ and $5 \cdot 10^{-4}$ M, after which a plateau was reached. Considering that the plasma concentration of Ca^{2+} is 1.5 mM and that the enzyme is localized on the outside of the cell, these data seem to indicate that enzyme activity is not regulated by the availability of Ca^{2+} [84].

A considerable purification (1020-fold) has recently been described for a phospholipase A_2 from rabbit platelets [85]. Heat treatment of platelet sonicates at pH 5, followed by extraction of particulate material with 1 M KCl, solubilized about 50% of the alkaline phospholipase activity. Further purification was achieved through gel filtration and CM-cellulose chromatography. No detergents were required to keep the enzyme in soluble form throughout these steps. The purified enzyme preparation was not yet homogeneous. The authors stated that the final preparation contained only a negligible amount of phospholipid, but the actual data, i.e., 1.4 nmol phospholipid per μg protein, are about twice the phospholipid-to-protein ratio found in most biomembranes.

The molecular weight of the rabbit platelet phospholipase A_2 is at variance with the molecular weight of 44 000 reported recently for a homogeneous phospholipase A_2 from human platelets [314]. The latter enzyme was purified by a two-step procedure employing solubilization from the membranes with H_2SO_4 followed by affinity chromatography. An overall enrichment of about 1300-fold was obtained by these procedures. On the other hand, Franson et al. [315,316] have obtained preparations of the membrane-associated phospholipase A_2 from human platelets which were 3500-fold purified compared with platelet homogenates. The molecular weight of their isolated phospholipase A_2 was estimated to be about 15 000 (Franson, R.C., personal communication). The reason for this discrepancy is currently unknown.

In summary, the purified phospholipases listed in Table III clearly show A_2 specificity and produce equimolar amounts of 1-acyl lysophospholipid and free fatty acid. All enzymes require Ca^{2+} for their activity, although the latter varies widely from one enzyme to the other. Most of the proteins have molecular weights comparable to the pancreatic phospholipase A_2.

IV. How is the activity of membrane-bound phospholipases A regulated?

With the availability of purified phospholipases A derived from intracellular membranes it is possible to examine several obvious questions. Is the phospholipase an integral or a peripheral membrane protein? On which side of the membrane is the enzyme, or at least its active site, located? How much of the protein is buried in the lipid bilayer? Can the membrane-associated enzyme only hydrolyze phospholipids in one monolayer of a biomembrane or does it have access to the phospholipids in both monolayers? If so, is this achieved through protein movement in the transverse plane of the bilayer or through transmembrane movement of the phosphoglycerides? The asymmetric distribution of phospholipids and the transmembrane movement of these constituents in biomembranes have recently been reviewed [86,87]. Perhaps the most important question is the aspect of regulation of the phospholipase activity. The membrane-associated phospholipases can be viewed as enzymes floating in a sea of substrate. What factors prevent and initiate digestion of cellular phospholipids in vivo? It is not very likely that general answers to

these questions, which apply to all membrane-associated phospholipases, can be given. One is therefore faced with studies of special cases and one has to attempt to abstract general regulatory mechanisms from these studies. Helpful in these respects are a large number of observations, which show an activation of phospholipase A activity as the consequence of a wide range of stimuli or treatments (see subsections IVB, C and E for specific examples). In these circumstances, the experimentally observed increase in phospholipase activity leads to the question: what changes at the molecular level augment enzyme activity? Unfortunately, our present knowledge about such activation phenomena is scanty and the description thereof has largely been given in terms of models by analogy with other enzyme systems, the regulation of which is better understood in terms of molecular events. Such models have to consider conversion of inactive zymogens to active enzymes, other covalent modification of the enzyme molecule, interaction with regulatory ligands or proteins and changes in membrane structure resulting in variations in the catalytic capacity of membrane-associated enzymes. The merits of such models and their experimental support will be discussed in the following sections.

IVA. Do intracellular phospholipases occur in zymogen form?

The actual occurrence of zymogens of phospholipase A_2 was demonstrated in a most straightforward manner by isolation of the precursor molecule from porcine pancreas by de Haas et al. [88]. Conversion of the zymogen into the active enzyme occurred by trypsin-catalyzed removal of a heptapeptide from the N-terminus of the molecule. This conversion could also be brought about by prolonged autolysis of a pancreatic homogenate at room temperature. This procedure resulted in a 10-fold increase in the phospholipase activity in the crude homogenate. The enzyme purified from autolyzed homogenates [74] was indistinguishable from the one obtained by trypsin activation of the zymogen [88].

Moderate activation (2–4-fold) of phospholipase A activity has been described in various rat tissues [89], but the positional specificity of the enzyme(s) involved was not studied. The activation process was found in cytosol fractions prepared from spleen, lung, thymus, liver and bone marrow. It was claimed that when homogenates of these tissues were submitted to centrifugal fractionation, 80% or more of the phospholipase activity with or without trypsin treatment was found in the supernatant fraction after centrifugation for 4 h at 120 000 × *g*. For liver, these results with non-treated homogenates are certainly at variance with the data reviewed in Section II, which indicated that the major part of the neutral or slightly alkaline phospholipase A activity is membrane-bound.

In early studies, the search for a phospholipase activity in erythrocytes failed to detect such an enzyme in the red blood cells of various animals. On the other hand, lysophosphoglycerides were the main acceptors during fatty acid incorporation in erythrocytes. This led to the belief that acylation of lysophosphoglycerides played an important role in the renewal of fatty acids in the phospholipids of these cells. Originally, the red cell was thought to be dependent on the plasma as a source of lysophosphoglycerides, an idea supported by experiments showing rapid exchange of lysophospholipids between plasma and erythrocytes. The ability of red cells to produce lysophospholipids themselves was first demonstrated by Paysant et al. [90] in rat erythrocytes, albeit with a phosphatidylglycerol, i.e., a non-erythrocyte phospholipid, as substrate. In contrast to rat erythrocytes, those of humans showed barely detectable phospholipase activity, which could only be demonstrated in lysates of human cells. Interestingly, treatment of the lysates with tryp-

sin resulted in an at least 10-fold increase in phospholipase A activity [91]. By several criteria, this increased activity was shown not to be due to a possible phospholipase in the trypsin preparation used, and thus suggested a trypsin-catalyzed activation of the erythrocyte phospholipase. The question as to whether this activation is caused by a zymogen-active enzyme conversion or by removal of an inhibitory peptide in response to trypsin treatment was not further investigated. At present, it is not even clear whether the observed activation is to be ascribed to the proteolytic activity of trypsin. Zwaal et al. [82] have been unable to detect phospholipase activity in human red cell ghosts, either before or after trypsin treatment. Increased phospholipase A_2 activity towards phosphatidylglycerol after trypsin treatment has also been observed in rat plasma [92]. A similar, 2–3-fold, increase was observed during storage of diluted plasma at 0°C for several days. After this activation, no further increase was obtained by trypsin treatment. Upon ethanol fractionation of whole rat plasma, the active enzyme appeared in fraction I, whereas the albumin-rich fraction V showed only phospholipase A activity after treatment with trypsin. The authors have concluded from these data that rat plasma contained an inactive precursor of phospholipase A which became transformed into an active enzyme with different solubility properties under the influence of either a plasma factor or trypsin [92]. Although a considerable purification of the active enzyme was reported [93], no attempt was made to isolate the precursor molecule and to show the activation in a system containing pure components. Other mechanisms to account for the activation process can therefore still be envisaged. Progress in this area has been hampered by a later observation [94] that the activation of plasma phospholipase A by the crude commercial preparation of pancreatic trypsin was due to the presence in this preparation of an activating factor which is different from trypsin itself. This may explain why Zwaal et al. [82], presumably using pure trypsin preparations, were not able to confirm the activation of human erythrocyte phospholipase A_2.

In addition to crude trypsin, intact or lysed rat platelets also activated the phospholipase A in platelet-deficient plasma [94]. Addition of rat platelets, likewise, caused a tremendous increase, varying from 6- to 35-fold, in the phospholipase activity of human serum and plasma, rabbit plasma and lysed human erythrocytes. The activating factor could be solubilized from rat platelets by a variety of techniques including sonication, repeated freeze-thawing and treatment with sodium deoxycholate or 0.1 M $CaCl_2$ at pH 4. The platelet factor appeared to be sensitive to heat and completely lost its activating property after 10 min at 60°C. The platelet lysate has since been used by Polonovski and colleagues [95,97] to activate a number of other phospholipases. Torquebiau-Colard et al. [95] have shown that rat liver plasma membranes have a low phospholipase activity with phosphatidylethanolamine as substrate which could be stimulated 5-fold by adding platelet lysate. Perfusion of the liver with saline prior to fractionation of the tissue yielded a plasma membrane fraction devoid of phospholipase activity, but after addition of platelet lysate the plasma membrane phospholipase activity was restored to the level present in plasma membranes of non-perfused livers in the presence of platelet lysate. By using liver plasma membranes of heparinized rats from which most of the phospholipase A_1 has been detached (Section II), it has been subsequently shown that most of the increase in total phospholipase activity can be ascribed to activation of a phospholipase A_2 [39]. The inactive phospholipase A_2 remained bound to the membranes when these were washed with 1 M NaCl. In contrast, activation of the phospholipase A_2 by the platelet factor prior to the washing procedure led to a complete solubilization of the active enzyme. These data suggest that activation is accompanied by a conversion of a

more hydrophobically bound integral membrane protein into a more electrostatically bound peripheral membrane protein [39]. As discussed by the authors two possible mechanisms may account for these observations. Schematically, these are depicted in Fig. 1. First, proteolytic action by the platelet factor could convert a prophospholipase into an active enzyme. Secondly, complex formation between the inactive phospholipase and the platelet factor could yield an active phospholipase. Unfortunately, no definitive distinction between these two possibilities can be made at present. It may be relevant, however, in this respect, to mention some recent experiments on the release of phospholipase A and triglyceride lipase from perfused rat liver or from isolated hepatocytes [96]. Although in most experiments total phospholipase activity was not differentiated into A_1 and A_2 activity, it was claimed that hepatocytes released a mixture of both enzymes, with phospholipase A_2 accounting for about one-third of the total phospholipid-hydrolyzing activity. The total activity released into the medium was 20-times greater than that present in the cells and this was not altered by puromycin concentrations which completely shut off protein biosynthesis. These data suggest the conversion of inactive to active forms of both phospholipases prior to or during release.

An inactive form of phospholipase A that could be converted into an about 2-fold more active form by addition of lysed platelets also has been described in fetal and adult rat lung [97].

Phospholipase A_2 has been postulated to play a regulatory role in the biosynthesis of prostaglandins [98] (but compare subsection VA) by releasing free arachidonate as a substrate for the cyclo-oxygenase system. Treatment of intact cells with proteolytic enzymes, in several cases, gave rise to increased release of arachidonate. Both thrombin and trypsin induced phospholipase activity in human platelets [99], suggesting a proteolytic modification of a phospholipase. However, compared with intact platelets, disrupted platelets were much less responsive to thrombin and trypsin. Thus, it would appear that the structural integrity of the platelet is indispensable for proteolytic enzymes to activate the phospholipase. Remarkably, EGTA was able to augment the effect of thrombin but not that of trypsin, suggesting that each enzyme influenced different points in the pathway leading to phospholipase activation. The above-mentioned fact that EGTA enhances the thrombin effect indicates that EGTA has no access to the intracellular Ca^{2+} required in the active site of the phospholipase A_2. This would seem to indicate that at least the active site of the phospholipase is located in the inner monolayer of the platelet membrane. It is still conceivable that part of a prophospholipase could be reached by the proteolytic enzymes from the outside to yield an active enzyme. If this were the sole event responsible for the activation, one would expect that activation could have been simulated by treatment of platelet membranes with proteolytic enzymes. Since this was not observed, an alternative explanation was postulated in that EGTA treatment of intact cells would chelate surface Ca^{2+}, thereby facilitating thrombin proteolysis. As with trypsin, this proteolytic attack of the membrane could perhaps have activated the phospholipase in a non-specific manner by altering membrane structure. Similar explanations can be put forward for the observed stimulation of arachidonate release, measured as prostaglandin production, in a transformed mouse BALB/3T3 cell line by thrombin and trypsin. Several other proteases (chymotrypsin, collagenase, elastase and thermolysin) did not stimulate prostaglandin production [100].

In conclusion, except for pancreatic phospholipase A_2, the occurrence of inactive zymogens of intracellular phospholipases has not been demonstrated convincingly at the molecular level. This would require the isolation of a, at least partially, purified pro-

enzyme, which can be converted by proteolysis into an active enzyme under conditions which unequivocally show a modification of the covalent structure of the proenzyme. It is obvious that studies on the activation of membrane-bound phospholipases, although interesting and of utmost importance in themselves, are not likely to provide an answer to this question. In the proteolytic activation of membrane-bound phospholipases it will be very difficult to unravel specific zymogen-active enzyme conversions from other possible activation mechanisms such as those due to non-specific alterations in membrane structure and proteolytic removal of inhibitory proteins. Before discussing the evidence for the presence of proteins or peptides which specifically regulate phospholipase A activities, the difficulty of interpreting proteolytic activation of membrane bound enzymes is further illustrated in the following examples. Adenylate cyclase levels in a rat embryo fibroblast cell line were found to be much higher in cells detached by trypsinization as compared to cells obtained by mechanical scraping [101]. The latter cells showed an 8-fold increase in cyclase activity upon trypsin treatment. A 2-fold activation of rat liver plasma membrane adenylate cyclase by several pure proteolytic enzymes was reported and explained by unmasking of active sites [102]. In contrast, proteolytic activation of adenylate cyclase in several cultured fibroblasts seemed to be more in line with an effect on the regulatory functions of the enzyme rather than on the catalytic unit [103]. In these cells, several other membrane-associated enzyme activities such as 5'-nucleotidase and both Mg^{2+}- and $(Na^+ + K^+)$-ATPase were not appreciably modified by trypsin treatment. This was used as an argument to indicate that proteolytic activation of the cyclase system is specific and is not the result of general alterations in membrane structure, thereby denying the well known fact that not all membrane-associated enzymes are equally sensitive to membrane perturbations. These results are only mentioned to indicate that phospholipase activation by proteolytic enzymes has its analogies in other areas of membrane research.

Finally, it should be noted that activation of phospholipases by conversion of zymogen into active enzymes represents an irreversible modulation of enzymatic activity. For physiological control processes, reversible regulation mechanisms would seem to be much more appropriate.

IVB. Are phospholipases regulated by specific association with non-enzymatic proteins?

As discussed in the previous section, the activation of phospholipase activities observed in a number of cases upon treatment with proteolytic enzymes has been explained by at least two general mechanisms, i.e., zymogen-active enzyme conversions or proteolytic inactivation of a non-enzymatic inhibitor of the phospholipase. Since no data on the molecular events resulting in activation are available it has been impossible to distinguish between these possibilities. This section deals with the evidence for the presence of proteins or peptides specifically modifying phospholipase activities. Inhibition of enzymatic activities by protein inhibitors is best known for proteolytic enzymes, where the inhibiting protein acts by binding stoichiometrically to the enzyme [104]. Does this type of regulation occur with phospholipases as well?

In their search for mutants of *Bacillus subtilis* with fragile membranes, Kent and Lennarz [105] isolated a mutant which, in contrast to wild-type cells, showed up to 70% phospholipid degradation during protoplast formation. This appeared to be due to the sequential action of a membrane-associated phospholipase A_1 and a cytoplasmic lysophospholipase. In subsequent experiments to explain why no comparable phospholipid degradation took place in wild-type protoplasts, it was found that wild-type cells con-

tained a potent inhibitor of the phospholipase A_1 in mutant cells. This inhibitor, apparently absent in the mutant, was present in both soluble and membrane-associated form in wild-type cells. The soluble form was heat-stable, non-dialyzable and sensitive to trypsin. Further evidence for the protein nature of the inhibitor was provided by Krag and Lennarz [106]. The soluble form of the inhibitor, located in the periplasmic space, was purified over 2000-fold to homogeneity by classical protein-purification techniques. Its estimated molecular weight amounted to about 30000 in gel filtration and about 36000 in SDS-urea gel electrophoresis experiments. Inactivation of the membrane-bound phospholipase A_1 appeared to occur via an enzymatic inactivation process rather than via stoichiometric binding of the inhibitor to the enzyme. Addition of purified inhibitor to mutant cells during protoplast formation not only prevented hydrolysis of membrane lipids bu so restored the osmotic stability of the mutant protoplasts, indicating a correlation between reduced membrane lipid content and increased fragility. On the bases of these results, it was postulated that the wild-type cell would contain a phospholipase A_1 as well, but that the expression of its activity would be masked by the inhibitor protein. This idea was corroborated by the finding that not only mutant cells but also wild-type cells secreted the lipolytic enzyme into the growth medium. In addition, several properties of the partially purified extracellular enzyme from wild-type cells were found to be identical with those of the partially purified lipolytic enzyme obtained from mutant membranes [107]. Both enzymes were not specific for phospholipids, but hydrolyzed diglycerides and diglucosyldiglycerides at slightly lower rates. The partially purified membrane-bound and extracellular (phospho)lipases were inactivated in an identical fashion by the inhibitor protein. The strong similarity between the membrane-bound and extracellular form of the (phospho)lipase suggested that the membrane-bound form might be an intermediate in the secretion of the (phospho)lipase. This hypothesis was supported by the finding that under appropriate conditions, about 60% of the (phospho)lipase activity was lost from the membranes of intact cells and an equivalent amount appeared in the medium. If the membrane-bound (phospho)lipase is in fact a secretion intermediate, then the role of the protein inhibitor in the wild-type cells could be to protect the membranes of the cell against the action of the (phospho)lipase during or after secretion. It should be recalled, however, that there is no evidence that the inhibitor protein exerts its irreversible inactivation of the (phospho)lipase through formation of a stoichiometric (phospho)-lipase-inhibitor protein complex.

Other evidence for the involvement of proteins in the regulation of intracellular phospholipase A_2 activity has recently emerged from studies on prostaglandin release. It is generally accepted that prostaglandins are not stored within cells but are biosynthesized in response to various stimuli. The rate-limiting step in the biotransformation of arachidonic acid into endoperoxide and further metabolites is believed to be a release of the precursor acid from complex cellular lipids by phospholipases [108–110] or other acyl hydrolases (see subsection VA). Prostaglandins have been implicated in inflammation and non-steroidal aspirin-like anti-inflammatory drugs like aspirin and indomethacin inhibit prostaglandin generation by inhibition of the cyclo-oxygenase enzyme [111]. Also, anti-inflammatory corticosteroids have been shown to interfere with prostaglandin production in, for example, perfused guinea-pig lungs [112] and transformed mouse fibroblasts [110]. Since corticosteroids had no appreciable effect on the cyclo-oxygenase in vitro, it was suggested that these anti-inflammatory agents acted by reducing the availability of intracellular substrate. Indeed, addition of hydrocortisone to fibroblasts did not inhibit prostaglandin formation from exogenously supplied arachidonic acid but instead reduced

the release of arachidonic acid from the cellular phospholipids [110]. Similarly, perfusion of phosphatidylcholine, labeled with oleate in the *sn*-2-position, through isolated lungs allowed the detection of a phospholipase A_2 activity which was inhibited by anti-inflammatory steroids [113]. It was then discovered that inhibition of prostaglandin formation in renal papillae by corticosteroids was completely abolished in the presence of inhibitors of RNA and protein biosynthesis, suggesting that synthesis of a protein factor was involved in the inhibition of prostaglandin production [114]. However, no direct measurement of phospholipase A_2 activities was made in these studies. Flower and Blackwell [115] were the first to provide evidence that protein synthesis was required for inhibition of the phospholipase A_2 in perfused lungs by corticosteroids. Perfusion of lungs with dexamethasone markedly reduced phospholipase A_2 activity as estimated by the release of thromboxane A_2 or by hydrolysis of radioactive phosphatidylcholine. During subsequent perfusion without dexamethasone, control levels of phospholipase activity were reached within 1 h, indicating a reversible inactivation. In the presence of inhibitors of

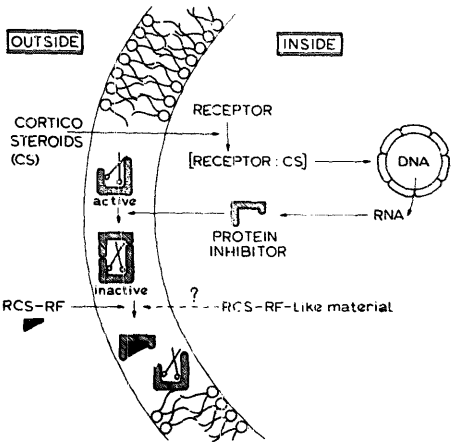

Fig. 2. Tentative scheme for the regulation of phospholipase A_2 activity by endogenous inhibitor and activator peptides. The following sequence of events is schematically depicted. Corticosteroids induce the synthesis in lung of a protein which somehow is involved in the inactivation of a phospholipase [115]. The depicted complex formation of active enzyme and inhibitor protein to yield an inactive complex is only one of several possible, as yet unresolved, mechanisms. Rabbit aorta contracting substance-releasing factor (RCS-RF) has been described as a compound of peptide nature, capable of stimulating phospholipase A_2 activity [125] when perfused through lungs. At present, the depicted action of RCS-RF, i.e., dissociation of the inhibitor protein from the inactive phospholipase A_2 inhibitor complex to yield active phospholipase A_2, should only be considered as one of several hypothetical modes of action. Stimulation of phospholipase activity in transformed mouse fibroblasts depends on protein synthesis [129,130], perhaps resulting in the production of RCS-RF-like material acting from inside the cells.

RNA and/or protein biosynthesis, dexamethasone perfusion failed to inhibit phospholipase activity. The perfusate of dexamethasone-treated lungs, but not that of untreated lungs, was shown to contain a factor which inhibited the phospholipase A_2 of a second lung. In view of the above-mentioned effect of protein synthesis inhibitors, this factor is likely to be a peptide or protein [115]. This experimental approach will eventually allow isolation of the factor so that its mode of action can be studied. At present, the results can most easily be explained by assuming that the factor reversibly modifies the phospholipase A_2, possibly by combining with the enzyme to yield an inactive enzyme-factor complex (Fig. 2). However, an alternative explanation in which the phospholipase is kept in an active form through association with a second factor, X, is also compatible with the experimental results, provided it is the factor X which is subject to modification by the protein synthesized in response to the anti-inflammatory steroids.

The previous paragraphs have dealt with the possibility of phospholipase A inhibition through interaction with proteins. In the following paragraphs the evidence for stimulation of phospholipases by non-enzymic proteins will be reviewed. The possibility of this occurring has clearly been demonstrated for several highly purified phospholipases or lipolytic enzymes in vitro, although even in these cases the exact molecular mechanisms underlying the activation process remain as yet unresolved. Lipoprotein lipases not only hydrolyze primary acyl ester bonds in triglycerides, but also those in phospholipids, thus expressing phospholipase A_1 activity. The enzyme can be released from the vascular endothelial surface by intravenous injection of heparin or during perfusion with heparin. Characteristic of the purified enzyme is that both its lipase and phospholipase A_1 activity [116] are greatly stimulated by apolipoprotein C-II, a peptide consisting of 78 amino acids. Similar activations have been described for the action of bovine milk lipoprotein lipase towards phosphatidylcholine vesicles [117]. To explain this activation it may be of importance to note that high affinity of apolipoprotein C-II for both phospholipid vesicles [117] and lipoprotein lipase [118] has been demonstrated. The apolipoprotein may thus function to link the enzyme to its substrate. While the exact mechanism is unclear, the finding that maximal activation was obtained when a stoichiometric 1 : 1 complex of lipoprotein lipase and apolipoprotein C-II had been formed strongly supports the hypothesis that this complex formation is the basis for the activation phenomenon [118].

Lecithin-cholesterol acyltransferase catalyzes the transfer of long-chain acyl groups primarily from the *sn*-2-position of phosphatidylcholine to cholesterol. The activity of the purified enzyme depends on the presence of an apolipoprotein from high-density lipoproteins [119]. Recently, the two partial reactions of lecithin-cholesterol acyltransferase, i.e., hydrolysis of phosphatidylcholine and esterification of cholesterol, have been uncoupled by demonstrating that the enzyme has major phospholipase activity when cholesterol is not present [120]. Not only the transferase activity, but also the phospholipase activity appeared to be completely dependent on the presence of apolipoprotein A-I. In this case, it was shown that the apolipoprotein A-I was not required for the binding of the enzyme to the liposomes, suggesting a direct activation of the phospholipase (and transferase) function of the enzyme through complex formation with the apo A-I.

Activation by non-enzymic proteins has been demonstrated also for hydrolysis of complex glycolipids by lysosomal hydrolases. In some cases, e.g., cerebroside-sulfatase [121, 122], the activating protein forms a 1 : 1 complex with the lipid substrate and this complex is then recognized as a substrate by the enzyme. In other cases, e.g., glucocerebrosidase [123], the activator forms a complex with the enzyme, when the latter is present in soluble form. The association between activator and enzyme reaches a 1 : 1 stoi-

chiometry when the activator concentration is limiting. Incorporation of the activator into a membrane preparation containing the catalytic protein was time-dependent and paralleled the appearance of enzyme activity in the membrane [124]. This indicates that activity of the membrane-bound enzyme is also affected by the activator. The lysosomal activators are relatively heat-stable, low molecular weight (about 25 000) acidic glycoproteins.

Some evidence for the existence of a peptide which stimulates phospholipase A_2 activity has been obtained by Nijkamp et al. [125]. It has long been known that anaphylaxis in isolated lungs from sensitized guinea-pigs leads to release into the perfusate of both an unstable rabbit aorta-contracting substance (RCS) and a much more stable RCS-releasing factor (RCS-RF). The latter releasing factor was able to promote a further release of RCS during perfusion of unsensitized lungs [126]. The main active component of RCS was later identified as thromboxane A_2, although some cyclo-endoperoxides were present as well [127,128]. RCS-releasing factor has been partially purified by Nijkamp et al. [125]. The almost 6000-fold purified material exhibited properties compatible with RCS-RF being a peptide of less than 10 amino acids. Hydrolysis of oleate-labeled phosphatidylcholine during perfusion of lungs was stimulated 2-fold by simultaneous injection of RCS-RF and this hydrolysis was blocked when steroids were present. These results suggested that RCS-RF activated a phospholipase A_2. Combination of these results with the steroid-induced synthesis of a phospholipase A_2 inhibitor leads to a tentative scheme for phospholipase A_2 regulation as indicated schematically in Fig. 2. The phospholipase can associate with an inhibitor protein to yield an inactive enzyme-inhibitor complex. Anti-inflammatory steroids enhance the synthesis of inhibitor molecules with the net effect of decreased phospholipase activity. Other peptides, such as RCS-RF, release the inhibitor from the inactive complex, perhaps through a direct interaction with the inhibitor, to give augmented phospholipase activity. Similar peptides may be synthesized in other organs and cells in addition to lung. Some evidence for this can perhaps be extracted from observations made by Levine et al. [129] and Pong et al. [130] on transformed mouse fibroblasts. Prostaglandin production by these cells is stimulated by serum, thrombin and bradykinin. This stimulation is blocked, however, by inhibitors of protein and RNA synthesis, albeit after a certain lag time. The requirement of protein synthesis for the expression of the stimuli suggests the synthesis of a peptide or protein factor which enhances prostaglandin production. Since the stimuli had no effect on the cyclo-oxygenase itself, it could be that the peptide factor, perhaps RCS-RF-like material, stimulated prostaglandin formation by providing more arachidonic acid substrate through stimulation of the phospholipase A_2. Alternatively, the observation could be explained by de novo synthesis of new phospholipase molecules. It should be remembered, therefore, that the scheme in Fig. 2 is highly tentative at present. Neither the inhibitor protein nor the activator protein has been isolated at present in sufficient purity to allow studies on their mode of action. Much work will have to be done to establish firmly the many hypothetical interactions put forward in this scheme.

IVC. Regulation of phospholipase A_2 by availability of Ca^{2+}

Membrane-bound phospholipases A_2 show an absolute requirement for Ca^{2+}. Kunze et al. [131] have found that prostaglandin biosynthesis in homogenates of bovine seminal vesicles is markedly stimulated by Ca^{2+}. Since Ca^{2+} slightly inhibited prostaglandin synthesis from free arachidonate but stimulated phospholipase A_2, these findings supported

the assumption that phospholipase A_2 plays an important role in stimulating prostaglandin biosynthesis by providing free precursor acids. In studying the patterns of fatty acid release from endogenous substrates by human platelet membranes, Derksen and Cohen [132] discovered a lipolytic enzyme releasing arachidonate. An equivalent amount of arachidonate was lost from the phospholipid fraction, thus suggesting that the lipolytic enzyme was a phospholipase A_2. Addition of Ca^{2+} augmented arachidonate release 20-fold. If these and other calcium-dependent phospholipases A_2 indeed function as regulators in intracellular archidonate release for endoperoxide synthesis one might expect that events which increase the availability of Ca^{2+} to the phospholipase A_2 in intact cells might stimulate the formation of endoperoxides and related products. Several research groups have provided evidence to support this hypothesis by using ionophores to elevate cytoplasmic Ca^{2+} at the expense of intracellular storage compartments, perhaps mitochondria [133] or the dense tubular system [134]. Pickett et al. [135] demonstrated that when washed human platelets were incubated in the presence of 5 mM EGTA, addition of the calcium ionophore, A 23187, led to the sudden release of arachidonate in much the same way as that produced with thrombin. In the presence of 10 μM ionophore, 46% of the total phospholipid arachidonate content was released within 1 min. The stimulatory effect of the ionophore was maximal in the presence of 5 mM EGTA and declined progressively as the concentration of free Ca^{2+} in the medium was raised. These data imply that in intact cells the EGTA did not penetrate to the locus of phospholipase A_2, which therefore is likely to be not at the outside surface. Independently, Rittenhouse-Simmons and Deykin [136] confirmed and extended these observations by showing that the abrupt release in prelabelled cells was from phosphatidylcholine and phosphatidylinositol. Interestingly, in cells deprived of metabolic ATP by preincubation with deoxyglucose and antimycin A, the release of arachidonate as promoted by thrombin, but not that by ionophore, was inhibited compared to control cells. Others have found that the thrombin-induced burst in oxygen utilization by platelets, an indicator of free arachidonate oxygenation, is markedly diminished by antimycin A. This suggested also that thrombin-induced release of arachidonate was largely dependent on electron transport and, presumably, ATP synthesis [137]. This impairment of the thrombin-induced arachidonate release in ATP-depleted cells was found even in the presence of extracellular Ca^{2+}, indicating that thrombin alone does not render the membrane sufficiently permeable to Ca^{2+} to account for the phospholipase activation [136]. The combined data were interpreted to indicate that a Ca^{2+} flux, resulting in a rise in cytoplasmic free Ca^{2+} concentration, activated phospholipase A_2. This flux of intracellular Ca^{2+} could be brought about either by the ionophore or by thrombin. It was proposed only in the latter case that a contractile or alternative ATP-dependent process is required to liberate internally stored Ca^{2+}. In this case, no covalent modification, such as, e.g., zymogen-active enzyme conversion (subsection IVA), would be necessary for thrombin-induced phospholipase activation [136]. It should be mentioned, however, that this explanation is completely based on the assumption that ionophore- and thrombin-induced activation share an identical last step, namely saturation of pre-existing enzyme with Ca^{2+}. The availability of free cytoplasmic Ca^{2+} for the phospholipase supposedly is controlled by cyclic AMP (compare next section and Fig. 3).

Ionophore stimulation of platelet thromboxane B_2 formation and cyclo-oxygenase oxygen consumption was not restricted to the divalent cationophore A 23187. The mono- and divalent cationophore, X 537A, produced comparable increases, while the monovalent cationophores, nigericin, monensin A and valinomycin, had no effect [137].

An interesting new dimension to the concept that platelet phospholipase A_2 is regulated by the availability of intracellular Ca^{2+} to the enzyme locus was recently added by Wong and Cheung [138]. These investigators presented some preliminary evidence suggesting that the stimulation of human platelet phospholipase A_2 by Ca^{2+} could be mediated through calmodulin, a ubiquitous Ca^{2+}-binding protein.

Data suggesting that A 23187-induced stimulation of thromboxane or prostaglandin biosynthesis may be a more generalized phenomenon were obtained by Knapp et al. [137]. The ionophore produced this effect also in rat renal medulla, rat stomach and trachea, human lymphoma cells and guinea-pig polymorphonuclear leukocytes. Contrary to what has been observed with platelets, A 23187 only stimulated prostaglandin synthesis in renal medulla when Ca^{2+} was added to the external medium [137]. The stimulatory effects of Ca^{2+}, Mn^{2+} and Sr^{2+} on prostaglandin formation in rabbit kidney medulla slices in the absence of ionophores were found to correlate with their stimulatory effects on the release of arachidonate and linoleate from tissue lipids [139].

IVD. Influence of cyclic adenosine monophosphate

As mentioned in the previous section, Pickett et al. [135] noticed that dibutyryl cyclic AMP inhibited thrombin-, but not ionophore-induced, activation of platelet phospholipase A_2. Using different techniques, other investigators arrived at similar conclusions concerning the effect of dibutyryl cyclic AMP on thrombin-induced arachidonate release from platelet phospholipids. Thus, Minkes et al. [140] found that incubation of platelets with dibutyryl cyclic AMP before thrombin addition blocked the subsequent formation of oxygenated derivatives of arachidonate. In contrast, when arachidonate was added directly to platelets preincubated with dibutyryl cyclic AMP no inhibition of thromboxane A_2 formation was observed.

This demonstrated that dibutyryl cyclic AMP had no direct inhibitory effect on cyclooxygenase or thromboxane synthetase, a conclusion in conflict with findings of Malmsten et al. [141] but independently confirmed by Lapetina et al. [142]. The latter authors used horse platelets and a more direct measurement of phospholipase A_2 activity, i.e., the loss of radioactive arachidonate from phospholipids of prelabeled platelets. Not only dibutyryl cyclic AMP but also agents which elevated platelet cyclic AMP levels, such as cyclic AMP phosphodiesterase inhibitors [142] or adenylase cyclase stimulators, such as prostaglandin E_1 [140,158] or prostacyclin [142], were found to prevent the thrombin-induced deacylation of arachidonate-labeled phospholipids. Contrary to the initial observation by Pickett et al. [135], it has later been found that also A 23187-induced phospholipase A_2 activation is inhibited by prior incubation with dibutyryl cyclic AMP [143, 144] or cyclic AMP phosphodiesterase inhibitors [143]. Many of these effects depend apparently on the relative concentrations of stimuli used, as Feinstein et al. [158] have described conditions under which arachidonate release in the presence of ionophore was normal despite high cellular cyclic AMP levels. It remains to be elucidated by what mechanism cyclic AMP inhibits the release of arachidonic acid. Excluding more general effects of cyclic AMP on cellular metabolism and assuming that the arachidonate-releasing phospholipase is directly regulated by cyclic AMP, several possibilities still can be envisaged. Among these are direct binding of dibutyryl cyclic AMP or cyclic AMP to the enzyme, cyclic AMP-dependent phosphorylation of the phospholipase or an interacting protein resulting in inhibition of the phospholipase. Alternatively, cyclic AMP could regulate Ca^{2+} fluxes and stimulate the storage of Ca^{2+}, thereby diminishing Ca^{2+} availability to the

phospholipase. Evidence to support the notion that phospholipase activity in platelets is solely dependent upon the cytoplasmic levels of Ca^{2+} was provided by Rittenhouse-Simmons and Deykin [144]. They showed that thrombin-induced release of arachidonate from human platelet phosphatidylcholine was not only impaired by dibutyryl cyclic AMP but also by the intracellular calcium antagonist, 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate. Addition of external Ca^{2+} did not abolish these inhibitions. However, when the platelet phospholipase was activated by the divalent cationophore A 23187, the inhibition produced by either dibutyryl cyclic AMP or the calcium antagonist could be overcome by addition of external Ca^{2+}. In platelet lysates the phospholipase A_2 was stimulated by addition of Ca^{2+}, but neither A 23187 nor dibutyryl cyclic AMP exerted under these conditions the respective stimulatory or inhibitory effect observed in whole cells. This shows that dibutyryl cyclic AMP does not inhibit the phospholipase by direct binding to enzyme or substrate, a conclusion confirmed by others [203]. It was suggested [144,158] that in intact cells cyclic AMP may promote a compartmentalization of intracellular Ca^{2+}, thereby reducing cytoplasmic free Ca^{2+} and inhibiting phospholipase A_2 activity. It is worth noting in this respect that Käser-Glanzmann et al. [145] have shown that platelet vesicles, possibly derived from the dense tubular system, could concentrate Ca^{2+} when incubated in the presence of cyclic AMP, ATP and protein kinase. Furthermore, the accumulated Ca^{2+} could be released from the vesicles by A 23187. These findings allow for the model of platelet phospholipase A_2 regulation by the availability of

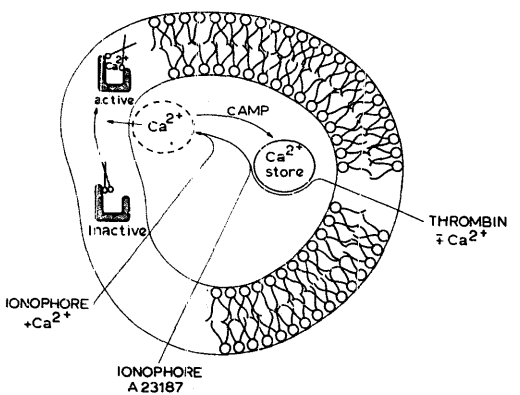

Fig. 3. Model for the regulation of platelet phospholipase A_2 by availability of Ca^{2+}. The conversion of an inactive phospholipase A_2 into an active phospholipase $A_2 : Ca^{2+}$ complex is depicted. A rise in cytoplasmic Ca^{2+} level can be induced by ionophore A 23187 either in the presence or absence of external Ca^{2+}. In the latter case, the Ca^{2+} is thought to be released from intracellular Ca^{2+} stores. Likewise, addition of thrombin is thought to lead to release of Ca^{2+} from this or another store in an ATP-dependent process. In the depicted model for the regulation of phospholipase A_2 activity, the inhibition observed by cyclic AMP (cAMP) is ascribed to its lowering of cytoplasmic Ca^{2+} levels.

Ca^{2+} as depicted schematically in Fig. 3. Thrombin or ionophore induce the release of Ca^{2+} from an intracellular store which activates the phospholipase A_2. Agents which enhance the intracellular cyclic AMP level or Ca^{2+} antagonists counteract the increase in cytoplasmic Ca^{2+} by sequestering the Ca^{2+}, thus resulting in an inhibition of thrombin- or ionophore-activated phospholipase A_2. The inhibition of the ionophore-, but not that of thrombin-induced phospholipase activation, by cyclic AMP can be overcome by external Ca^{2+}. Only in the former case does the extracellular Ca^{2+} gain access to the phospholipase, thus overruling the decrease in cytoplasmic free Ca^{2+} as produced by cyclic AMP. If indeed Ca^{2+} sequestering is an ATP-requiring process [145], the observation that ATP deprivation inhibits thrombin-induced phospholipase A_2 activation [136,137] is difficult to reconcile with this model, unless Ca^{2+} release is also energy-requiring and more sensitive to lowered ATP levels than sequestering of Ca^{2+}. On the other hand, the free cytoplasmic Ca^{2+} concentration as modulated by cyclic AMP may not be the only factor regulating phospholipase A_2 activity. In addition, there is no compelling evidence to show that phospholipase A_2 activation by ionophore follows the same mechanism as thrombin- or collagen-induced activation. In fact, Lindgren et al. [146] have recently reported almost complete inhibition of collagen-induced arachidonate release from platelet-rich plasma under conditions where intracellular cyclic AMP levels were not yet elevated. Differential inhibition of thrombin- and collagen- as compared to ionophore-induced phospholipase activation was reported by Feinstein et al. [158]. Using malondialdehyde formation as a measure for arachidonate release, it was shown that prostaglandin E_1, causing increased cyclic AMP levels, almost completely inhibited thrombin- and collagen-induced malondialdehyde formation, but had no effect on ionophore A 23187-stimulated malondialdehyde production. Although this would still be explainable by compensation for the cyclic AMP decrease in cytoplasmic Ca^{2+} with ionophore, other experiments demonstrated that phospholipase activation by thrombin or collagen, but not that by ionophore, was blocked by the protease inhibitor, phenylmethanesulfonyl fluoride. In the case of thrombin, kinetic evidence was provided to show that this effect was not due to inactivation of thrombin itself. On the basis of these results, it was postulated that two different mechanisms underlie the phospholipase activation in platelets depending on the stimulus [158]. Ionophore-induced stimulation would be due to Ca^{2+} activation of an existing active form of the phospholipase. If stimulation by collagen or thrombin fails to mobilize sufficient Ca^{2+} to stimulate maximally this active form, an additional mechanism is proposed to operate. The latter stimuli would activate a serine-protease, which in turn catalyzes the conversion of an inactive form of the phospholipase to an active form (compare subsection IVA).

Inhibition of prostaglandin biosynthesis by cyclic AMP appears to be a unique feature of platelets. In many other systems, including cultured mammalian cells, Graafian follicles, thyroid cells, adrenal cortex and adipocytes, a stimulation has been observed (see Ref. 147 for review and references therein). Therefore, the inhibition of phospholipase A_2 by cyclic AMP, either direct or indirect, as observed in platelets may not be of general occurrence. In thyroid, dibutyryl cyclic AMP, although stimulating prostaglandin release, was shown to have no effect on a phospholipase A_2 releasing arachidonate from phosphatidylinositol [148]. In contrast, indications of stimulation of phospholipases by cyclic AMP were obtained in lung [149] and adipocyte [150] homogenates. In the latter case, the use of doubly labelled phosphatidylethanolamine, allowing for a determination of the positional specificity of the phospholipases, and the complete dependency of the hydrolysis process on added Ca^{2+}, gave strong indication of the stimulation of a phospho-

lipase A_2 by cyclic AMP [151]. A similar conclusion was reached by Lindgren et al. (Ref. 153 and unpublished data) for 3T3 fibroblasts. Prostaglandin release from these cells was stimulated markedly in the presence of either dibutyryl cyclic AMP or N^6-butyryl-cyclic AMP, provided that theophylline was simultaneously added. Elevated levels of intracellular cyclic AMP, induced by adenosine and a phosphodiesterase inhibitor, preceded prostaglandin production. From prelabelled cells, dibutyryl cyclic AMP plus theophylline stimulated radioactivity release only in those cells labelled with homo-γ-linolenic acid or arachidonic acid. No such increased release over control cells was measured when the prelabelling was carried out with palmitate, stearate, oleate, linoleate or linolenate. These results would be compatible with the stimulation by cyclic AMP of a rather specific phospholipase A_2, which preferentially releases homo-γ-linoleate and arachidonate either through acyl-chain selectivity or substrate availability.

IVE. Effects of hormones on phospholipase activities

Although various hormones are known to stimulate prostaglandin synthesis or release in their respective target tissues (e.g., Table IV), only a few studies have appeared so far which have concentrated on the specific influence of hormones on the tissue phospholipases (Table V). Haye et al. [154], when studying the increased biosynthesis of prostaglandins in porcine thyroid slices discovered that thyreostimulin stimulated an endogenous phospholipase A_2. This enzyme hydrolyzed unsaturated fatty acids from exogenous phosphatidylcholine and phosphatidylinositol. Whereas the fatty acids released from phosphatidylcholine were mainly mono-unsaturated, phosphatidylinositol hydrolysis yielded mainly arachidonate. Since in thyroid slices both thyreostimulin and dibutyryl cyclic AMP stimulated prostaglandin biosynthesis, the possibility existed that thyreostimulin stimulation of the phospholipase A_2 was mediated by cyclic AMP. This possibility was made unlikely by the subsequent findings [148] that cyclic AMP had no effect on the phospholipase A_2 activity in thyroid homogenates under conditions where thyreo-

TABLE IV

HORMONAL STIMULATION OF PROSTAGLANDIN PRODUCTION

ACTH, adrenocorticotropin hormone; TSH, thyreostimulin; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Hormone	Target tissue	Reference (examples)
Angiotensin-II	kidney	165,166
	cultured mammalian cells	161,167
Bradykinin	heart	168
	lung	169
	kidney	162,171
	cultured mammalian cells	161,167,170
Epinephrine	spleen	172
ACTH	adrenal	173
TSH	thyroid	154,174
FSH	ovary	175
LH	ovary	175

TABLE V

HORMONAL STIMULATION OF LIPOLYTIC ENZYMES

Abbreviations as in Table IV; PA_2, phospholipase A_2; chol.est., cholesterol esterase; RMIC, renomedullary interstitial cells; cAMP, cyclic AMP.

Hormone	Target	Assay	Enzyme	Stimulation (-fold)	Implied mechanism	Reference
(A)						
TSH	thyroid	homogenate	PA_2	4	cAMP-independent	148,154
Prolactin	mammary gland	membranes	PA_2	2	?	157
Bradykinin	heart	perfusion	PA_2 or lipase	2.8	?	159
	kidney	perfusion	PA_2 or lipase	3.8	?	160
	RMIC	cells	PA_2 or lipase	36		161
	lung	perfusion	PA_2	2.3	?	175,176
	skin	membranes	PA_2	3.0	?	185
Angiotensin-II	kidney	perfusion	PA_2 or lipase	3.1	?	160
	RMIC	cells	PA_2 or lipase	72		161
(B)						
ACTH	adrenal	cytosol	chol. est. *	1.4	cAMP	177–179
	adrenal	cytosol	lipase *	1.4	cAMP	177
Epinephrine						
ACTH	adipose	homogenate or	lipase *	1.8–12	cAMP	180–184
TSH	tissue	cytosol				
glucagon						

* This lipase may be identical to the cholesterol esterase [182–184].

stimulin stimulated this activity 4-fold. Dibutyryl cyclic AMP stimulated, however, arachidonate release from triglycerides. It is therefore believed that thyreostimulin activates a phospholipase A_2 in thyroid by an as yet unknown, but cyclic AMP-independent, mechanism. Thyreostimulin also raises the cyclic AMP level [154,155], which activates a lipase. Both enzymes potentially can release arachidonate and thus contribute to the thyreostimulin-stimulated prostaglandin biosynthesis. Indeed, in the presence of albumin and indomethacin, thyreostimulin-stimulated thyroid slices were found to contain free arachidonate levels twice those of control slices [156].

Prolactin, when added to membranes prepared from mammary gland, effected a 2-fold increase in the release of arachidonate from exogenous, arachidonate-labelled, phosphatidylcholine [157]. The concentration of prolactin necessary to produce this effect was rather high, i.e., at least two orders of magnitude greater than those required to stimulate biochemical events in mammary gland explants. On the other hand, specificity of the prolactin effect was suggested by the observation that similar concentrations of growth hormone did not stimulate arachidonate release.

When hearts, prelabelled with arachidonate, were perfused, injection of bradykinin resulted in a 5-fold stimulated release of radioactive prostaglandin, without a significant increase in the amount of free arachidonate in the effluent. However, when fatty acid-free bovine serum albumin was added to the perfusate, the free arachidonate was apparently trapped before reincorporation into tissue lipids masked completely the release of free acid. Under these conditions, bradykinin administration caused an elevation in arachidonate in the cardiac effluent. The increase in fatty acid release upon hormone stimulation appeared to be a specific phenomenon for arachidonate-labelled hearts and was not observed with hearts prelabelled with linoleate, oleate or palmitate [159]. This selective bradykinin effect on fatty acid release suggests that an enzyme is activated that either distinguishes different fatty acids or is selectively compartmentalized with arachidonate-containing tissue lipids. Ischemia, on the other hand, exhibited a non-specific stimulation resulting in release of oleic as well as arachidonic acid. Since most of the labelled unsaturated fatty acids in the prelabelled heart were present at the 2-position of phospholipids, the activated enzyme(s) most likely belongs to the phospholipase A_2 type, but this was not proved by identification of lysophospholipids as reaction products, or by a correlation of released radioactivity with loss of label from the phospholipid pool. At the beginning of the experiments, the neutral lipid pool contained a substantial amount, about 15–20%, of the total radioactivity, so that activation of lipases cannot be excluded completely. It was noticed that during ischemia the ratio of released arachidonate to prostaglandins (10 : 1) was much higher than that found after bradykinin stimulation (3 : 1). This was interpreted to indicate that the hormone-stimulated deacylation of tissue lipids is more tightly coupled to the cyclo-oxygenase than that occurring during ischemia [159, 162]. Similar conclusions were reached by Schwartzmann and Raz [160] for bradykinin- or angiotensin-II-stimulated release of arachidonate and prostaglandin E_2 from rabbit kidney. These authors avoided the use of prelabelling procedures to exclude the possibility of selective release of radioactive acids from preferentially labelled pools. Approx. 5% of the arachidonate released under basal conditions was converted to prostaglandin E_2. Hormone stimulation not only resulted in a 2–3-fold selective increase in arachidonate release not seen for other fatty acids, but also led to a more efficient conversion into prostaglandin E_2, which now amounted to about 30%. The activated enzyme, apparently tightly coupled to the prostaglandin-generating system, was not further identified. This holds also for the acylhydrolase responsible for the large stimulation of arachidonate

release from prelabelled rabbit renomedullary interstitial cells in tissue culture as caused by bradykinin and angiotensin-II [161].

In view of the existing uncertainty as to which enzyme is actually activated, it is obvious that no rigorous data about the exact mechanism underlying these activations can be given. It is only for thyreostimulin-activated thyroid phospholipase A_2 (Table V) that some indications have been obtained which point to a cyclic AMP-independent activation mechanism. In all other systems, cyclic AMP could either directly or indirectly be involved. This holds especially for those cases where hormone-stimulated lipases, rather than phospholipases A_2, might be responsible for elevated arachidonate release. For comparison, some well known examples of cyclic AMP-mediated, hormone-stimulated, acylhydrolases are included in Table V. It should be noted, however, that some recent data appear to indicate that epinephrine-induced lipolysis in fat cells cannot be explained solely on the basis of cyclic AMP-dependent phosphorylation of the hormone-sensitive lipase. It has been suggested that hormone treatment leads to modification in the nature of the lipase-substrate interaction, possibly through interaction of epinephrine with phospholipids resulting in facilitated hydrolysis by the hormone-sensitive lipase [163,164].

IVF. Regulation of phospholipases by changes in membrane structure

It has recently been proposed [186] that activation of phospholipases might occur by changes in the structural arrangement of phospholipids in (plasma) membranes. That such a proposal is not merely speculative can be deduced from the large influences which membrane phospholipids can exert on the activity of membrane-bound enzymes (see Refs. 187 and 188 for recent reviews). For example, Sinensky et al. [189] have recently correlated adenylate cyclase activation with increased ordering of the acyl chains of membrane lipids. In contrast, reduction of the fluidity of phospholipid fatty acyl chains gave rise to a decrease in $(Na^+ + K^+)$-stimulated ATPase activity [190]. Much of the information of this type has been obtained using reconstituted systems in which the phospholipid acyl-chain and polar-headgroup composition can be varied at will. Such reconstituted systems for membrane-bound phospholipases are not yet available. It cannot be excluded that reconstitution in fact takes place during the assay of purified membrane-bound phospholipases A (Tables II and III), but this has not been proved rigorously. There is some indirect evidence which might suggest that membrane-associated phospholipase activity is influenced by the fluidity of the membrane. Local anesthetics can cause both membrane fluidization [191] and facilitated phospholipid cleavage [192,194]. The latter effect is strongly concentration-dependent and a direct correlation between fluidization and enzyme activity was not established.

The effect of detergents on the activity of membrane-associated phospholipases has been documented in numerous reports. In most cases, exogenous substrates were used and the detergent effect is most likely due to facilitation of the interaction of the membrane-bound enzyme with the dispersed lipids. In general terms, this can probably be accomplished by events such as loosening of the membrane structure, perhaps accompanied by a partial solubilization of enzyme or enhanced incorporation of the dispersed lipid substrate in the residual membrane structure. On the other hand, the detergents may only exert their effect at the substrate level by providing the enzyme with a substrate aggregate of mixed lipid-detergent micelles to which the enzyme has a greater affinity or penetration power resulting in enhanced catalytic capacity. An obvious difficulty is that

various parameters, e.g., surface charge, spacing of lipid molecules, fluidity of acyl chains and absorption of enzyme to the surface, as modified by the detergent may not be mutually independent. No systematic study unravelling these various possibilities has been carried out so far for membrane-associated phospholipases. Some analogies with much better defined systems using purified soluble phospholipases may be drawn here. The latter systems have been extensively reviewed by Verger and de Haas [9]. Especially from monolayer studies, it has become clear that three factors are particularly important in governing the activity of phospholipases added to lipid films, i.e., *zeta* potential on the substrate surface [195], packing of the lipid molecules and absorption of enzyme molecules to the surface followed by penetration into the lipid monolayer [196,197]. Thus, densely packed phosphatidylcholine monolayers were not attacked by phospholipase A_2, unless dicetyl phosphoric acid was also present [198]. Although this result could be explained by the increased distance between adjacent lecithin molecules, similar enhanced hydrolysis was not observed when stearylamine was used as spacer. Apparently, a negative surface charge is of utmost importance in the initiation of this phospholipase (*Naja naja*) activity. Enhanced activity, also of membrane-bound phospholipases, has often been reported by addition of anionic detergents, e.g., bile acids. In some cases, the negative charge provided by bile acids to the mixed-micelle surface appears to be an important factor in itself as similar effects can be obtained by including negatively charged phospholipids in the substrate aggregate. It is possible then, that membrane phosphorylation resulting in an increased negative charge might influence the regulatory membrane environment of a phospholipase. It should be realized, however, that it is highly questionable whether effects observed for the attack of lipid monolayers or aggregates by soluble phospholipases can be extrapolated to situations where both enzyme and substrate are part of the same membrane. A similar situation exists for the influence of the packing of the phospholipid molecules in the membranes. Monolayer studies have clearly shown [196,197] that a surface pressure can be created above which the lipolytic enzymes phospholipase A_2 and lipase can no longer penetrate the lipid film. Below a surface pressure of about 30 dyne/cm for pancreatic phospholipase A_2, there appears to be an optimum in the velocity of enzyme action as a function of surface pressure. In elegant experiments this was shown to be the result of at least two counteracting factors [197]. With increasing surface pressure the amount of enzyme in the interface decreased, while on the other hand the specific activity of the interfacial enzyme increased with the closer packing of the substrate molecules at higher surface pressures. That the packing of lipid molecules in the substrate aggregate plays an important role in the initial attack by phospholipases was also demonstrated in bulk experiments. Hydrolysis of artificial lipid bilayers, prepared from well defined molecular species of phosphatidylcholine, was strongly enhanced at the phase transition temperature. It was concluded that the coexistence of solid and liquid phases in the bilayer created irregularities in lipid packing at the border of these domains facilitating the penetration of the phospholipase A_2 [199]. Similar conclusions were reached for a 1020-fold purified preparation of rabbit platelet phospholipase A_2 [85]. Extrapolating from such observations it is tempting to speculate that membrane-associated phospholipase activities could be enhanced by any event that creates irregularities in the bilayer arrangement of phospholipids. Again, however, the experimental conditions employing soluble phospholipases added to lipid substrates are essentially different from those occurring in natural membranes. In this case, enzyme and substrate are present in the same membrane to begin with, thus precluding effects of facilitated penetration. These differences stress the need mentioned earlier in this section for a reconstituted sys-

tem of a membrane-associated phospholipase A_2 to enable a systematic study of the influence of the structural arrangement of the membrane lipids on phospholipase activity.

V. Specific functions of intracellular phospholipases

VA. Release of prostaglandin precursors

The role of acylhydrolases in the release of precursor-free fatty acids for prostaglandin formation was firmly established during the last decade. Prostaglandins do not occur in stored form in tissue phospholipids [98]. Conversion of arachidonate from phospholipid into prostaglandins does not take place unless the arachidonate is first released by addition of a phospholipase A_2 [200–202]. Once formed, the prostaglandins cannot be reincorporated into phospholipids under conditions that allow efficient incorporation of arachidonate [151]. These and other experiments convincingly demonstrated that the cyclo-oxygenase is limited in its production of prostaglandins by the amount of precursor-free fatty acid. It was logical to assume then, that endogenous acylhydrolases fulfilled an important role in providing free fatty acid from endogenous lipids. Much work has since been carried out to answer the question as to which lipid class serves as the exclusive or most important store of the arachidonate released prior to prostaglandin production. On a quantitative basis, the *sn*-2-position of phospholipids is, by far, richest in arachidonate, suggesting that the enzyme involved in arachidonate release was a phospholipase A_2. Much of the evidence for this involvement of phospholipase A_2 has been discussed in the previous subsections of Section IV. Especially in more intact biological systems, such as perfused organs, often only fatty acid release was measured. Thus, it was not always clear whether arachidonate release was exclusively from phospholipids and, if so, from which phospholipid class(es) and by which mechanisms. This problem will not be discussed here in general terms, but will be considered in detail in the system which has been investigated most intensively, i.e., platelets. A recent review [204] on the role of lipids in platelet function has dealt with data published until 1977.

The possible pathways for arachidonate release in platelets are summarized in Fig. 4. One of the earliest studies on lipolytic activities in platelets was carried out by Smith and Silver [205]. Using specifically labelled phosphatidylcholines they detected a phospholipase A_1 activity, which was optimal at pH 4.8 and required taurocholate. Treatment of human platelets with thrombin released the enzyme into the medium. These characteristics supported the belief that the enzyme was of lysosomal origin. The same enzyme has also been detected in rabbit platelet sonicates [203]; Ca^{2+} did not affect the activity. The substrate specificity of this enzyme has not been investigated. As indicated in Fig. 4, this enzyme can only contribute to arachidonate release in conjunction with a lysophospholipase. The latter enzyme, optimally active at pH 8.5, was reported to be present in rabbit [203] and human [31] platelets. At pH 4.5 the lysophospholipase activity was almost completely abolished. This and the finding that the optimal phospholipase A_1 activity at pH 4.5 amounted to only about 25% of that of a Ca^{2+}-requiring phospholipase A_2 activity at pH 9.5 have directed much attention to the latter enzyme as a likely candidate for arachidonate release in platelets.

Studies on the substrate specificity of this phospholipase A_2 have not yet provided a clear picture. Depending on whether endogenous or exogenous phospholipids were used, different results for the preference for phospholipid class and acyl chain at the *sn*-2-posi-

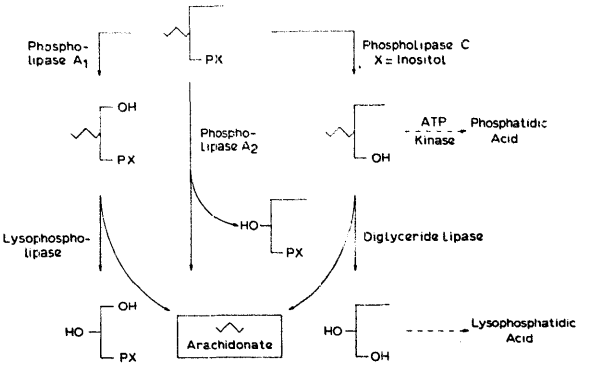

Fig. 4. Possible pathways for arachidonate release in platelets.

tion were reported for this phospholipase A_2 (compare Table VI). When human platelets were labelled with arachidonate in platelet-rich plasma, subsequent treatment of washed platelets caused a major decrease in the radioactivity of phosphatidylcholine and phosphatidylinositol [206,207]. Similar patterns for arachidonate release were noticed when washed platelets were prelabelled [208] or when the phospholipase A_2 was stimulated

TABLE VI

SUBSTRATE SPECIFICITY OF PLATELET PHOSPHOLIPASE A_2

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

Species	Preparation	Substrate	pH	Contribution to fatty acid release (%)				Reference
				PC	PE	PI	PS	
Human	prelabelled cells	endogenous	7.4	70	0	25	5	206
	prelabelled cells	endogenous	7.4	78	7	11	4	207
	prelabelled cells	endogenous	7.4	68	0	32	0	208
	prelabelled cells	endogenous	7.4	arachidonoyl PC species only, all species of PI + PS, no PE species				211
Horse	prelabelled cells	endogenous	7.4	31	34	35	–	142
Rabbit	prelabelled cells	endogenous	7.4	42	22	29	7 *	209,210
				14	58	25	3 **	209,210
Human	membranes	endogenous	9.5	28	69	<3	<3	132,217
Rabbit	membranes	exogenous	9.5	preference for PE, no preference for fatty acid				203

* Release of radioactive arachidonate.

** Release of total arachidonate.

with ionophore A 23187 [144]. In contrast, phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine contributed about equally to the thrombin-induced arachidonate release from prelabelled horse platelets [142,143]. Remarkably, when the cells were treated with ionophore A 23187 rather than with thrombin to induce phospholipase A_2, radioactively labelled arachidonate was only lost from phosphatidylcholine and phosphatidylinositol, but no longer from phosphatidylethanolamine [143]. It is presently unknown whether this can be used as an argument for the presence of different activatable phospholipases A_2. Some of the contrasting findings with regard to the question as to which phospholipid(s) donate free arachidonate after phospholipase A_2 stimulation can, at least, partially be explained by the different extents to which the various phospholipid classes incorporate arachidonate during prelabelling [142,143,206,207]. Hardly any labelling of phosphatidylserine was observed in horse platelets [142], thus excluding the possibility to measure arachidonate release from this phospholipid. Only minor amounts, if any, of arachidonate were released from phosphatidylserine and phosphatidylethanolamine in intact human platelets, despite the fact that about 6 and 12% of the labelled arachidonate had been incorporated in these phospholipids, respectively [206,208]. Blackwell et al. [209,210] emphasized the disadvantages of using prelabelled platelets in which the specific radioactivity of the arachidonate may vary with the phospholipid class. When the collagen-stimulated release of radioactive arachidonate from prelabelled rabbit platelets was measured, 41.6% originated from phosphatidylcholine, 28.7% from phosphatidylinositol, 22.5% from phosphatidylethanolamine and 7.2% from phosphatidylserine. When the pool sizes of arachidonate in the phospholipids as deduced from gas chromatographic analysis were taken into consideration, it could be calculated that phosphatidylcholine contributed 14.3%, phosphatidylinositol 24.6%, phosphatidylethanolamine 57.7% and phosphatidylserine 3.4% to the total arachidonate release. Two assumptions were made in these calculations. First, the phospholipase A_2 does not differentiate between [1-^{12}C]arachidonate and [1-^{14}C]arachidonate. This assumption is generally made in radioactive-tracer studies and in all likelihood is valid. The second assumption is that complete mixing of phospholipid species containing unlabelled arachidonate with the corresponding species containing [1-^{14}C]arachidonate has taken place with respect to availability for phospholipase A_2 hydrolysis. The validity of this assumption is much less likely and at least should be verified experimentally. In other words, the possibility exists that a small pool of, e.g., phosphatidylethanolamine becomes heavily labelled during the prelabelling procedure, perhaps because of its localization in the plasma membrane or certain areas thereof. Then, the percentage hydrolysis measured for this small pool does not necessarily apply to the total platelet phosphatidylethanolamine pool and calculations based on this assumption may very well yield erroneous results. The validity of such an assumption should be checked independently by showing that the specific radioactivity of the phospholipid class does not change during phospholipase A_2 action. In this light, the conclusion that most of the arachidonate, released during collagen-induced aggregation of rabbit platelets, originates from phosphatidylethanolamine should still be considered tentative. Apparently, species differences contribute to the variability as to which phospholipid contributes most to arachidonate release. Thus, phosphatidylethanolamine is not hydrolyzed in human platelets [206–208,211], while it is in rabbit [209,210] and horse [142,143] platelets.

Further detailed studies on the specificity of the phospholipase A_2 in human platelets were reported by Bills et al. [211]. Using cells prelabelled with various unsaturated fatty acids, phosphatidylcholine hydrolysis upon thrombin treatment was only found in arach-

idonate-labelled platelets. This suggested that the phospholipase A_2 which utilizes phosphatidylcholine is specific for the arachidonoyl-containing species. In support of this notion, a 25.6% decrease in radioactivity from phosphatidylcholine was accompanied by a 7.6% loss of phosphatidylcholine phosphorus, in agreement with analytical data indicating that human platelet phosphatidylcholine consists of about 24–30% of arachidonoyl species [213]. Arachidonate release was also observed from the phosphatidylinositol + phosphatidylserine fraction, but not from phosphatidylethanolamine. In contrast to the specific release of arachidonate from phosphatidylcholine, hydrolysis of phosphatidylinositol and/or phosphatidylserine was also noticed in cells radioactively labelled with oleate or linoleate. Due to much less efficient incorporation of these acids in comparison to arachidonate, the total release of radioactivity amounted to only a few percent of that observed with arachidonate. Corroboratively, when the amounts of non-radioactive fatty acids were measured only arachidonate was seen to accumulate in response to thrombin [211]. Such data suggest a selective mechanism for arachidonate release either through acyl chain specificity of the phospholipase A_2 involved or through compartmentalization of the substrates in relation to the enzyme. Acyl chain specificity cannot be excluded, but it should be noted that this has generally not been found with other phospholipases A_2. In addition, such specificity is not found with platelet membrane fractions in combination with exogenous substrates (see below). Compartmentalization of enzyme and substrate appears to be, therefore, a more likely explanation for the specific release of arachidonate upon thrombin stimulation of human platelets. It becomes interesting then to review the scarce data on phospholipid and arachidonate localization in platelets.

Chap et al. [214] estimated that 63% of the phospholipids in pig platelets are located in the plasma membrane. The composition of those plasma membrane phospholipids is 26% sphingomyelin, 30% phosphatidylcholine, 27% phosphatidylethanolamine and 16% phosphatidylinositol + phosphatidylserine. From a comparison of the degradative action of *N. naja* phospholipase A_2 and *Staphylococcus aureus* sphingomyelinase on lysed and intact platelets, it was concluded that the outer monolayer contained 46% of the total plasma membrane phospholipids. This layer contained 91% of the sphingomyelin, 40% of the phosphatidylcholine, 34% of the phosphatidylethanolamine and less than 6% of the phosphatidylinositol + phosphatidylserine in the plasma membrane. Similar distributions were found in human platelets, except that somewhat less of the phosphatidylethanolamine was present in the outer plasma membrane leaflet. This appears to be compensated by higher percentages of phosphatidylcholine and phosphatidylinositol in the outer monolayer [215]. These data extend observations made by Schick et al. [216] who used trinitrobenzenesulfonate to label aminophospholipids. In intact human platelets, no phosphatidylserine reacted with the probe, while phosphatidylethanolamine labelling leveled off at 12–17% in reasonable agreement with the 12% of the total platelet phosphatidylethanolamine which could be hydrolyzed by exogenous phospholipase A_2 under non-lytic conditions [214,215]. What bearing do these phospholipid localizations have on the observed selective hydrolysis of phosphatidylcholine and phosphatidylinositol by the thrombin-stimulated phospholipase A_2 of human platelets? One straightforward explanation could be given by assuming that the phospholipase A_2 is located in the outer monolayer of the plasma membrane where it hydrolyses phosphatidylcholine and phosphatidylinositol. This necessitates the further assumption that the small amount of phosphatidylethanolamine in the outer monolayer either is not available for the enzyme or does not contain arachidonate. Two arguments at least can be provided against this reasoning.

Firstly, evidence is available which seems to argue against a localization of the phospholipase on the outer surface of platelets (discussed in subsection IVA). Secondly, although about 60% of the platelet phospholipids is thought to be present in the plasma membrane [214,215] with about 30% then being in the outer monolayer, studies on the distribution of total arachidonate have led to the conclusion that only 10% thereof is located in the outer surface monolayer. Thus, the outer surface seems to be rather impoverished in arachidonate. On the other hand, if the platelet phospholipase A_2 is located in the inner leaflet of the plasma membrane or in an intracellular membrane it is not obvious, at present, why specifically phosphatidylcholine and phosphatidylethanolamine are attacked, considering the fact that most of the arachidonate-containing other phospholipid species are present at these cellular sites as well. Compartmentalization of the enzyme and substrate would then have to be proposed as the basis for the observed selective degradation of phospholipid classes or species. In all likelihood, such compartmentalizations occur in the intact platelet prior to and during stimulation of the endogenous phospholipase A_2. At the same time, it should be realized that the experimental evidence to support such compartmentalizations is very poor indeed.

Other experiments to elucidate the substrate specificity of the platelet phospholipase A_2 have employed isolated total membrane fractions rather than intact cells. Derksen and Cohen [132] incubated isolated membranes in the presence of Ca^{2+} and determined gas-chromatographically the amount of fatty acid released from endogenous substrates. Optimal release of arachidonate was observed at pH 9.5, while palmitate, stearate and oleate release peaked at pH 8.5. This led the authors to postulate the presence of phospholipase A_1 and A_2 activities with optima at pH 8.5 and 9.5, respectively. It cannot be concluded with certainty, at the moment, whether the optimal release of saturated fatty acids and oleate at pH 8.5 is due to the presence of a phospholipase A_1 or to the presence of phospholipase A_2 plus lysophospholipase [203]. At pH 9.5, the increase in free arachidonate was accompanied by an equivalent loss of arachidonate from the phospholipid fraction. The putative phospholipase A_2 responsible for this release would be expected to release linoleate with equal facility as this acid shows a propensity similar to arachidonate for esterification at the *sn*-2-position of phosphoglycerides. In fact, it was observed that arachidonate and linoleate were released in a molar ratio of 12–15 : 1 whereas the phospholipids of freshly isolated platelet membranes contained these fatty acids in a molar ratio of about 6 : 1 [132]. In a subsequent, more detailed study it was shown that these molar ratios in phosphatidylcholine and phosphatidylethanolamine amounted to 1.7 : 1 and 17.8 : 1, respectively. This suggested that, in the absence of any acyl chain specificity of the phospholipase A_2, phosphatidylethanolamine would be the preferred substrate. Direct measurement of the arachidonate contents of the phospholipids and free fatty acid chains before and after incubation corroborated this suggestion. Of the total arachidonate released, 28% originated from phosphatidylcholine, 69% from phosphatidylethanolamine and only about 3% was released from sources other than these two phosphoglycerides [217]. Using a particulate fraction isolated from rabbit platelet sonicates and exogenous labelled substrates, Kannagi and Koizumi [203] similarly arrived at the conclusion that phosphatidylethanolamine was the preferred substrate. Under these conditions, the enzyme did not exhibit strict acyl chain specificity. In fact, hydrolysis of 1-acyl-2-[1-^{14}C]oleoyl phosphatidylcholine was slightly higher than that of 1-acyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine. It remains to be established why in isolated platelet membranes phosphatidylethanolamine is the preferred substrate [217], while in intact human platelets no breakdown of this phospholipid is observed [206,207,211].

Two possible explanations for this discrepancy come to mind. The phospholipid degradation in intact platelets is always measured after stimulation, usually by thrombin. Does the basal phospholipase A_2, as assayed in isolated membranes, show a different substrate specificity from that of the thrombin-stimulated phospholipase A_2? The other difference which should not be overlooked is that the membranes were incubated at pH 9.5, while studies with intact platelets were performed at pH 7–7.4. It may be relevant to mention, in this respect, that a partially purified phospholipase A_2 from rat liver mitochondria hydrolyzed phosphatidylethanolamine more rapidly at pH 9.5 than at pH 7.4, whereas the reverse was true for phosphatidylserine [55].

In most studies with either platelets or membranes thereof, attention was focussed on fatty acid release. At best, this was correlated with a decrease in the fatty acid under consideration in the phospholipids or in a given phospholipid class [132,211,217]. This correlation in itself does not prove the action of a phospholipase A_2, as similar results would be expected for the conjunctive action of a phospholipase C and a lipase (compare Fig. 4). It is therefore important to note that the presence of phospholipase A_2 in platelet membranes has been demonstrated unequivocally through isolation of both fatty acid and lysophospholipids [203,217]. Jesse and Cohen [217] analyzed the lysophosphatidylethanolamine that was produced from endogenous phosphatidylethanolamine along with the fatty acid release and showed that it contained virtually no fatty aldehydes. This led the authors to conclude that exclusively diacyl phosphatidylethanolamine, but no plasmalogen phosphatidylethanolamine, was attacked by the phospholipase A_2. It was not stated, however, whether accumulation of lysophosphatidylethanolamine was stoichiometric with phosphatidylethanolamine breakdown. It should be considered that rapid incorporation of arachidonate from phosphatidylcholine and phosphatidylethanolamine into plasmalogen phosphatidylethanolamine has been reported in human platelets [207]. Thus, the paucity of fatty aldehydes in the isolated lysophosphatidylethanolamine could also be due to a selective reacylation of the plasmalogen lysophosphatidylethanolamine.

That the above mentioned possibility of the concerted action of phospholipase C and lipase could explain both fatty acid release and phospholipid breakdown in intact platelets is not purely hypothetical and has been reported by several investigators. Rittenhouse-Simmons [212] demonstrated that human platelets generate diglycerides within 5 s of exposure to thrombin to yield up to 30-fold increased levels. This diglyceride accumulation was transitory and within 2 min the cells had essentially achieved the low levels of diglyceride characteristic of the resting state. In cells prelabelled with arachidonate within the time course of diglyceride accumulation, only phosphatidylinositol showed a sizable loss of radioactivity, which could easily account for the gain in diglyceride. Other potential diglyceride precursors, either did not lose radioactivity (triglycerides) or showed an increased amount of label (phosphatidic acid). Phosphatidylethanolamine and phosphatidylserine did not lose radioactivity in the examined period, while phosphatidylcholine started to show significant loss of labelled arachidonate only after 30 s and continued to do so well after the time that diglyceride had returned to normal levels. These data strongly suggested that phosphatidylinositol was the source of the diglyceride produced. In line with this conclusion, a phosphatidylinositol-specific phosphodiesterase was discovered in the soluble fraction of platelet sonicates. This phospholipase C-type enzyme hydrolyzed phosphatidylinositol to form diglyceride and inositol phosphate, but did not produce diglyceride from phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine. The presence of this enzyme in platelets was independently discovered also by Mauco et al. [213] and Bell et al. [219]. The enzyme required Ca^{2+} [212,218]

and, when assayed with exogenous phosphatidylinositol, apparently a negatively charged lipid surface. The enzyme was active only in the presence of deoxycholate [212] or when phosphatidylinositol concentrations exceeded 50 μM [218]. Unfortunately, no data are yet available to explain the sudden activation of this phosphatidylinositol-specific phosphodiesterase upon thrombin treatment of platelets. Interestingly, Rittenhouse-Simmons [212] demonstrated that pretreatment of platelets with dibutyryl cyclic AMP completely inhibited both diglyceride production and serotonin release. Dibutyryl cyclic AMP, which is thought to act, in part, to restrict Ca^{2+} mobilization, could thus prevent the activation of the phosphodiesterase, which in the absence of dibutyryl cyclic AMP might be caused by making stored Ca^{2+} available to the enzyme in much the same way as has been proposed for the platelet phospholipase A_2 (compare subsection IVC). A similar conclusion was reached by Billah et al. [318].

Bell et al. [219] have examined the fatty acid composition of the accumulated diglyceride, which consisted of almost equimolar amounts of stearate and arachidonate, in line with the fatty acid composition of platelet phosphatidylinositol [213]. Furthermore, in confirmation of preliminary data obtained by Mauco et al. [220], they also demonstrated the presence of diglyceride lipase in the particular fraction of platelets. This enzyme could generate the arachidonate for the burst of prostaglandin and thromboxane synthesis that follows thrombin stimulation. Thrombin stimulation releases about 5–10 nmol of arachidonate per 10^9 platelets. Since the activity of the cytosolic phosphodiesterase amounted to 15–20 nmol phosphatidylinositol hydrolyzed/min per 10^9 cells [218] and that of the particulate diglyceride lipase to 31 nmol fatty acid released/min per 10^9 cells [219], it follows that the activity of these enzymes is sufficient to account for the arachidonate release. It should be pointed out, however, that the above-mentioned activities were measured with saturating substrate levels, which may not necessarily apply to the in vivo situation. This holds especially for the low levels of transitory diglyceride as substrate for the lipase. On the other hand, the involvement of a phosphatidylinositol-specific phospholipase C and a diglyceride lipase in arachidonate release from stimulated platelets offers an explanation for several findings observed shortly after thrombin activation. Firstly, that only arachidonate is released upon thrombin stimulation may be explained by the fact that the diglyceride substrate originates from phosphatidylinositol and contains virtually only arachidonate in the *sn*-2-position. The release of arachidonate and other unsaturated fatty acids from other phospholipids by an activated phospholipase A_2 would in this view be a secondary event following the burst of phosphatidylinositol degradation after thrombin addition. Secondly, transient diglyceride accumulation followed by phosphorylation of the diglyceride could explain the increased levels of phosphatidic acid repeatedly observed in platelets after thrombin stimulation [143, 212,220]. This indicates that the diglyceride lipase is not the only enzyme in platelets that acts on the diglyceride. The diglyceride lipase in platelet membranes showed little, if any, activity towards triolein [219]. Also, in another aspect, this lipase is unique in that it apparently removes fatty acids from the *sn*-2-position. Most other lipases studied so far show an overwhelming preference for the acyl ester bond at the primary hydroxyl group and fatty acids from the *sn*-2-position are only hydrolyzed after acyl migration. Such a non-enzymatic step is difficult to accept in a mechanism which regulates arachidonate release from platelet lipids. When the particulate platelet fraction was incubated with diglyceride labelled with ^3H in the glycerol backbone and ^{14}C in the fatty acid at the *sn*-2-position, no radioactive monoglyceride was detected [219]. This indicates that the enzyme hydrolyzes stearate from the *sn*-1-position as fast as arachidonate from the *sn*-2-

position. Alternatively, other enzymes in the particulate fraction might prevent the accumulation of monoglycerides. Two possibilities come to mind. A monoacylglycerol hydrolase has recently been described for human platelets [221]. This enzyme has not been assayed with diacylglycerol as substrate. Several properties seem to be rather similar to the diglyceride lipase described by Bell et al. [219]. The possibility of both enzymatic activities residing in a single protein seems worthy of investigation. Whether or not the diglyceride lipase and the monoglyceride lipase are the same or enzymes acting in sequence, this pathway does not explain why upon thrombin stimulation only arachidonate and no stearate is released. This could, perhaps, be explained by the presence of a second enzyme preventing the accumulation of monoglyceride, i.e., monoglyceride kinase. The latter enzyme, perhaps identical to diglyceride kinase, as well as the accumulation of lysophosphatidic acid during thrombin-induced platelet aggregation have been described by Mauco et al. [220]. These authors also provided evidence to show that the lysophosphatidic acid was not formed from phosphatidic acid through phospholipase A action. Obviously, many of the enzyme specificities in this recently discovered phospholipase C plus diglyceride lipase pathway for arachidonate release remain to be established.

VB. Phospholipid turnover

Phospholipids have long been viewed as static building blocks of subcellular structures. As in many areas of biochemistry, an extra dimension was added to this structural role of lipids when radioactive isotopes became available. The early experiments employing ^{32}P showed that the phospholipids of all tissues became rapidly labelled. This rate of labelling of tissue phospholipids was hardly influenced by hepatectomy, except that under these experimental conditions no labelling of plasma phospholipids occurred [222]. These and many other experiments using isolated organs, tissue slices or homogenates have established the view that virtually all tissues are more or less autonomous in their phospholipid synthesis. Not only was a rapid labelling of the phospholipids observed after a pulse with radioactively labelled phosphate, but the incorporated radioactivity disappeared rapidly again from the phospholipid fraction. These observations have led to the concept of phospholipid turnover, in which a constant supply of newly synthesized phospholipids is balanced by a removal of existing molecules. The half-lives of the total phospholipid pool or of specific phospholipid classes in only a few tissues or cell-types are summarized as examples in Table VII. A consideration of the data in Table VII clearly shows the complexity of the turnover phenomenon. Relatively short half-lives of disappearance of labelled phospholipids are noticed in all tissues, except for brain, notably the myelin fraction (Expt. 5). Considerable variation is found in dependence of the precursor used (compare Expt. 3 with 6 and the results for lung in Expt. 8). This has to be ascribed most likely to extensive re-utilization of the labelled atoms, thus giving rise to falsely high values for the half-life of the component under investigation. The early experiments have often used the disappearance of label from the total phospholipid pool of whole tissues. Although such experiments were adequate to demonstrate the dynamic character of tissue phospholipids, it has since been realized and demonstrated that the individual phospholipid classes turnover independently and at quite different rates (Expts. 5–7, 16). Even within a given phospholipid class, the different molecular species which comprise that class turnover at different rates (Expt. 9). Turnover studies in whole organs can obviously be influenced strongly by secretion of intact phospholipid molecules. This certainly contributes

a problem in studies with liver, which is known to synthesize plasma and bile phospholipids. This raises the question as to whether the phospholipids in the liver itself turn over at all. This has been studied by isolation of liver subcellular membranes at various periods after pulse-labelling. The data in Expts. 10–14 of Table VII clearly demonstrate that phospholipid turnover occurs in all subcellular membranes. In accordance with expectation, the observed half-lives are considerably longer, however, than found in whole liver (compare Expts. 1–4 and 10–14). Although considerable variation in the data can be noticed, it seems justified to conclude that the glycerol-phosphate-base (hydrophilic) backbone in the membrane phospholipids turns over faster than the hydrophobic acyl chain part of the molecules (Expts. 10 and 14 as opposed to 12 and 13). This points to a more extensive re-utilization of acyl chains when compared to the glycerol-phosphate-base backbone.

It is an interesting and, as yet, unresolved question as to why turnover of phospholipids, constituting metabolic endproducts, should be necessary for the cell's physiology. Provocative ideas on this subject have been formulated by Dawson [242], who considered natural death and autolysis of cells, movement of cell membranes in exo- and endocytosis processes and maintenance of structural integrity of cellular membranes to support membrane functions as processes which might have some bearing on the phenomenon of phospholipid turnover. Arguments have since been put forward which seem to suggest that replacement of dead cells in tissues through cell division and cell growth is not the only mechanism underlying phospholipid turnover. Studies with cells in tissue culture have shown that phospholipid turnover was similar for growing and non-growing cells under conditions where no secretion of phospholipids into the medium took place [239,241]. With mouse fibroblasts [239], metabolic inhibitors which suppressed the synthesis of phospholipids caused an equivalent decline in the rate of degradation. Thus, the anabolic and catabolic events of turnover appear to be tightly coupled in these cells. Increased turnover of acidic phospholipids, notably phosphatidic acid and phosphatidylinositol, during phagocytosis or hormone stimulation of various glands (see Ref. 243 for review) is usually accompanied by metabolic stability of the main phospholipids phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, during the period of investigation. The most likely reason for phospholipid turnover then appears to be that it sustains membrane integrity and membrane function. Such a vague conclusion is not very satisfactory, however, since it in no way explains the necessity for continuous synthesis and degradation of phospholipids. A plausible explanation could be, as suggested by Dawson [244], that turnover is required to replace damaged phospholipid molecules due to auto-oxidation of the polyunsaturated fatty acid chains in phospholipids. It has recently been shown that lipid peroxidation of membrane suspensions in vitro leads to increased phospholipid bilayer rigidity, which in turn can cause impairment of membrane functions [245]. If auto-oxidation were the main reason for turnover, one would expect shorter half-lives for the more susceptible (poly)unsaturated phospholipid species and appreciable metabolic stability for the diunsaturated phospholipids.

Clearly, this is not the case (Table VII, Expt. 9). Trewhella and Collins [246] have likewise concluded that of all the phosphatidylcholine molecules present in rat liver, the 1-stearoyl-2-arachidonoyl species had the slowest rate of turnover. We are, therefore, faced with the problem of being unable to provide a compelling reason as to why phospholipid turnover is required.

In this review I shall not deal with the biosynthetic part of this turnover. A few remarks on the catabolic sequence of turnover seem appropriate in a review on phospho-

TABLE VII

TURNOVER OF PHOSPHOLIPIDS

Abbreviations as in Table VI. Δ0, Δ1, Δ2 and Δ4, disaturated, monoenoic, dienoic and tetraenoic species, respectively; mito, mitochondria; OM, outer membrane; IM, inner membrane; micros., microsomes; PM, plasma membrane; BHK, baby hamster kidney.

Expt.	Tissue	Precursor	Tissue fraction	Phospholipid class	Half-life (h)	Reference
(1)	Rat liver	[^{32}P]phosphate	total	total	6 –10	225–227
(2)	Dog liver	[^{32}P]phosphate	total	PC	1.8– 3.0	223
(3)	Rat liver	[^{32}P]phosphate	total	PC	10.9	224
(4)	Rat liver	[^{14}C]choline	total	PC	10	229
(5)	Rat brain	[^{32}P]phosphate	mito	PC	340	228
				PE	680	
				PI	50	
			myelin	PC	1350	
				PE	4750	
				PI	850	
(6)	Rat liver	[^{14}C]serine	total	PC	1.2	230
				PE	2.1	
				PS	7.0	
(7)	Rat intestine	[^{14}C]serine	total	PE	0.4	230
				PS	1.0	
(8)	Rabbit lung	[^{32}P]phosphate	total	PC	88	231,232
		[^{3}H]choline		PC	13	
		[^{3}H]glycerol		PC	8	
		[^{14}C]palmitate		PC	10	

(9)	Rat liver	[^{2}H]ethanol		PC Δ0	12	233
				Δ1	0.7–1.8	
				Δ2	2.5–4.0	
				Δ4	7.8–18	
(10)	Rat liver	[^{14}C]glycerol	micros.	total	44	234
				backbones	29	
				acylchains	140	
(11)	Rat liver	[^{32}P]phosphate	mito	total	38	235
(12)	Rat liver	[^{3}H]glycerol	mito	total	16	237
			micros.	total	16	
(13)	Rat liver	[^{14}C]choline	mito	PC	15	236
			OM	PC	16	
			IM	PC	3	
			micros.	PC	8	
(14)	Rat liver	[^{14}C]acetate	micros.	total	48	238
				PC	48	
				PE	48	
			PM	total	48–72	
				PC	48	
				PE	48	
(15)	Mouse fibroblast	[^{14}C]glucose	total	total	24	239
(16)	BHK cells	[^{3}H]glycerol	total	PC	4	240
				PE	4–6	
				PI	15	
(17)	Neoplastic mast cells	[^{14}C]choline	all organelles	PC	15–25	241

lipases A. It is generally thought that the catabolism of diacyl phosphoglycerides in most, if not all, cells proceeds through a consecutive deacylation catalyzed by phospholipases A_1 or A_2 and lysophospholipases. Phospholipase C activity is mainly found in some bacteria, while phospholipase D is mostly confined to the plant kingdom. A few exceptions to these general rules are known. A phospholipase C-type enzyme with absolute specificity for phosphatidylinositol is present in many mammalian cells, but its activity towards other phosphoglycerides has so far not been reported unequivocally. Phospholipase D has recently been partially purified from rat brain [247], but the role of this enzyme in turnover has yet to be established.

As early as 1955, Dawson [248] concluded from specific radioactivity versus time relationships that glycerophosphocholine and glycerophosphoethanolamine were intermediates in the catabolism rather than in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine in rat liver. The intracellular deacylating enzymes catalyzing these conversions have only later been detected (Section II). As might be expected for a consecutive deacylation pathway, the specific radioactivity of lysophosphatidylcholine was found to be intermediate between those of phosphatidylcholine and glycerophosphocholine [249]. The deacylation process apparently proceeds without any appreciable accumulation of the intermediary lysophosphatidylcholine, the amount of which appears to be kept rather constant at a level of about 0.5–1% of the total lipid phosphorus. This implies that the intracellular enzymes utilizing lysophospholipids, i.e., acyl-CoA : lysophospholipid acyltransferases and lysophospholipases should be able to cope with the production of lysophospholipids through phospholipase action. Over 80% of the lysophospholipase activity in rat liver is present in the $100\,000 \times g$ supernatant of homogenates [250]. The subcellular distribution of the total lysophospholipase activity varies considerably from tissue to tissue (described in Ref. 250). For bovine liver, it has later been demonstrated that the tissue contained at least two enzymes with lysophospholipase activity. Both enzymes were purified to homogeneity [251] and their individual subcellular localization was determined [252]. One enzyme appeared to be in the microsomal fraction, whereas the other lysophospholipase was mainly recovered in the cytosol. The presence of soluble lysophospholipases in both rat and bovine liver raised the question as to whether these enzymes can act on lysophosphatidylcholine embedded in membrane structures. Most likely, this is the form in which the lysophospholipid is present when produced during phospholipid degradation by intracellular membrane-bound phospholipases. Studies employing the purified bovine liver enzymes have shown that this enzyme is active against lysophosphatidylcholine embedded in both model membranes [253] and liver microsomal membranes [254]. Thus, the cytosolic enzyme is likely to contribute to the further catabolism of phosphoglycerides in those subcellular membranes that can be reached by the enzyme and in this way to function in preserving membrane integrity. A rigorous proof that these hypotheses apply to the in vivo situation could only be given if specific inhibitors were available that could be used in vivo to establish whether inhibition of the lysophospholipase would result in accumulation of lysophospholipids, and to what extent, in the individual subcellular fractions. The availability of such inhibitors would also allow an investigation of whether the anabolic and catabolic parts of turnover are coupled. In the absence of such inhibitors, results of in vitro experiments have to be extrapolated with caution to the in vivo situation. It is interesting to note that in vitro [253,254], the velocity of lysophosphatidylcholine hydrolysis increased almost linearly with the mole percentage of lysophosphatidylcholine in the membranes. This would tend to suggest that the cytosolic lysophospholipase acts under intracellular conditions, i.e.,

with lysophosphatidylcholine concentrations in the subcellular membranes of about 1% of the total phospholipids [255], far below its maximal activity. In this view, an increased concentration of lysophosphatidylcholine in such membranes may lead to enhanced activity of the lysophospholipase in an attempt to restore low lysophosphatidylcholine levels.

The role of lysosomal phospholipases (Section II) in phospholipid catabolism has recently been reviewed extensively [256]. It is interesting to note that when the phospholipids in isolated microsomal membranes were subjected to hydrolysis by lysosomal extracts, the above-mentioned pathways for phospholipid degradation were confirmed. Catabolism of phosphatidylcholine and phosphatidylethanolamine proceeded via a deacylation pathway with some accumulation of the respective lysophospholipid, while phosphatidylinositol was mainly degraded by the phospholipase C pathway [257]. It remains to be determined then whether the activity of intracellular phospholipases A_1 and A_2 is sufficient to account for the half-lives of disappearance given in Table VII. The enzymatic activities of the membrane-bound phospholipases discussed in Section II vary roughly from 1 to 10 nmol substrate hydrolyzed/min per mg protein. Most liver membranes contain about 600 nmol lipid phosphorus per mg protein, so that half of the membrane's phosphoglycerides can be degraded in a period of 30–300 min. Since the bulk of liver phospholipids occurs in membrane structures, even the most conservative estimate shows that the activity of the intracellular phospholipases is sufficient to account for the half-lives in Table VII. A valuable criticism against such calculations is that the specific activity of the membrane-bound enzymes was determined, in most cases, with saturating substrate concentrations. It seems more appropriate to use hydrolysis rates determined with endogenous substrates. Using this approach, Bjørnstad [258] observed in rat liver microsomes a disappearance of 10 and 50%, respectively, for phosphatidylcholine and phosphatidylethanolamine per h. The corresponding hydrolysis rates in mitochondria were about 20 and 50%, respectively [52]. Also, these values are not in conflict with the conclusion that the intracellular phospholipases A possess sufficient hydrolytic activity towards membrane-bound substrates to allow the turnover rates observed in vivo.

VC. Synthesis of molecular species

The phosphoglycerides of any cell comprise a heterogeneous mixture of individual molecular species due to the specific combination of saturated and unsaturated fatty acids. There is now a large body of evidence (reviewed extensively in Ref. 259) to indicate that not all molecular species are synthesized de novo. In this respect, the statement in Section VB that phospholipids are metabolic end-products to support membrane structure and function appears to be an oversimplification. The de novo synthesis of phosphatidylcholine in rat liver produces primarily monoenoic and dienoic species (e.g., see Refs. 260 and 261). Similarly, the specificity of the enzymes involved in the de novo synthesis of phosphatidylethanolamine is such that mainly dienoic and hexaenoic species are produced [260–262]. Both phosphatidylcholine and phosphatidylethanolamine are known to contain considerable amounts of tetraenoic species, with a saturated fatty acid at the *sn*-1- and arachidonate at the *sn*-2-position. Current opinion takes the view that arachidonate is mainly introduced by remodelling of de novo-synthesized species via a deacylation-reacylation mechanism. Briefly, this view is based on the following lines of evidence. Arachidonate tends to be excluded in the synthesis of phosphatidate [263,269]; the phosphoglycerides contain much higher percentages of arachidonate species than their immediate precursor, 1,2-diglycerides [264], and a selective utilization of diglyceride species

containing arachidonate by either choline phosphotransferase or ethanolamine phosphotransferase has been exluded [262,265]. The replacement of oleate and linoleate in de novo-synthesized monoenoic and dienoic phosphoglyceride species, respectively, by arachidonate through a deacylation-reacylation mechanism requires that at least part of the monoenoic and dienoic species cannot be considered as metabolic end-products. In this view, one would expect these species to show a higher turnover than the accumulating species with arachidonate. This has indeed been observed for both phosphoglycerides in in vivo experiments [233,261,266]. The half-lives of phosphatidylcholine monoenoic and dienoic species with palmitate as saturated acid were in the order of 1–2 h. The arachidonate species, on the contrary, showed half-lives of 4–21 h, depending on the saturated fatty acid present. It was noticed in all these studies that molecular species with palmitate turned over much more rapidly than species containing stearate. These and other observations [272] support the notion that also stearate is to a large extent incorporated via a deacylation-reacylation cycle.

Again, such remodelling mechanisms imply the action of phospholipases A_1 and A_2 on de novo-synthesized species to produce the 2-acyl and 1-acyl lysophospholipids as substrates for specific acyl-CoA : lysophospholipid acyltransferases. The presence of these phospholipases in liver has been amply discussed (Section II) and, indeed, the lysophosphatidylcholine fraction from rat liver has been demonstrated to consist of an almost equimolar mixture of the 1-acyl and 2-acyl isomers [7]. Very suggestive evidence for the operation of deacylation-reacylation cycles in vivo was obtained in dietary studies [267]. When essential fatty acid-deficient rats were shifted to a corn-oil diet, the rapid fall in eicosatrienoyl species of phosphatidylcholine was balanced by an increase of arachidonoyl species in an almost perfect mirror-image manner. This suggested the replacement of only the eicosatrienoic acid at the *sn*-2-position. The acyl-CoA : 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase in total rat liver has later been shown to acylate intravenously injected lysophosphatidylcholine preferentially with arachidonate [268]. The enzyme could be separated from other acyltransferases of rat liver microsomes [269] and has recently been obtained in a solubilized form with a 30-fold increased specific activity [270]. Both preparations showed a preferential incorporation of arachidonate into the acceptor, 1-acyl-*sn*-glycero-3-phosphocholine.

Additional arguments for the operation of deacylation-reacylation cycles in the biosynthesis of particular phospholipid species were provided by Kanoh and Akesson [271]. These authors prepared rat hepatocytes enriched in dilinoleoyl, dipalmitoyl or dimyristoyl phosphatidylcholine by pulse incubation of isolated cells with [^{3}H]glycerol and ^{14}C-labelled fatty acids. The cells were chased in tracer-free medium to follow the disappearance of the labelled species. The indicated species do not occur to any appreciable extent in the absence of the added fatty acids, so that their degradation could be studied without significant resynthesis of these species. About 40% of the dilinoleoyl, 70% of the dipalmitoyl and 30% of the dimyristoyl phosphatidylcholine were degraded during a 2 h chase. Data on the positional distribution of the labelled fatty acids during the chase demonstrated that the degradation of dilinoleoyl phosphatidylcholine and dilinoleoyl phosphatidylethanolamine proceeded through phospholipase A_1 action. In contrast, the degradation of dipalmitoyl phosphatidylcholine could be accounted for by the action of phospholipase A_2. A considerable part of the 2-linoleoyl and 1-palmitoyl glycerophosphocholine produced during the chase was reutilized for synthesis of the normal phosphatidylcholine species with saturated fatty acids at the *sn*-1-position and unsaturated fatty acids at the *sn*-2-position. The data clearly show the involvement of phospholipases A_1

and A_2 in mechanisms modulating the acyl chain composition of phosphoglycerides in intact cells, albeit so far only for rather unnatural species. It is worth noting, however, that the differential attack of diunsaturated species by phospholipase A_1 and that of disaturated species by phospholipase A_2 is in keeping with the production of lysophospholipid isomers that, through their specific acylation with saturated or unsaturated fatty acids by acyltransferases, will yield the naturally occurring phosphoglyceride species.

The involvement of phospholipases A_1 and A_2 in the remodelling of diunsaturated and disaturated phosphatidylcholines was suggested further by the finding that the degradation of these species was inhibited up to 95% when the chase was performed in the presence of 10 mM tetracaine [271]. However, the effect of local anesthetics on membrane-bound phospholipases is complex, with stimulation at low and inhibition at high concentrations (compare subsection IVE). Since the intracellular localization and concentration of tetracaine is unknown, its mode of action is difficult to assess. A more specific inhibitor for intracellular phospholipases would be highly desirable. Potentially, *p*-bromophenacyl bromide could serve this function for phospholipase A_2, although its effect in intact cells has not been studied systematically. In vitro experiments, however, demonstrated the usefulness of this inhibitor in studies on fatty acid elongation in brain mitochondria and microsomes [273]. Both fractions produced a C22 : 4 fatty acid as an elongation product of endogenous arachidonate. The first step in this elongation process is thought to be the release of arachidonate from the *sn*-2-position of membrane phosphoglycerides and subsequent utilization of the released arachidonate by enzymes of the elongation system. The elongated fatty acid may be reincorporated into phospholipids, thus providing another example of modulating the fatty acid composition of membrane phospholipids. In line with the above reasoning, it was found that *p*-bromophenacyl bromide inhibited strongly the elongation of endogenous fatty acids, while no effect was seen on the elongation of added fatty acids. These results demonstrate that *p*-bromophenacyl bromide inhibited the phospholipase A_2 without having an effect on the enzymes involved in the elongation process. The inhibitor is known to react with an active-site histidine in pancreatic [274] and snake venom [275] phospholipase A_2 and its inhibiting action on some other enzymes, therefore, cannot be excluded.

The incorporation of arachidonate via arachidonoyl-CoA : lysophospholipid acyltransferase is not limited to phosphatidylcholine and phosphatidylethanolamine. The labelling patterns of liver phosphatidylinositol species as a function of time after injection of either phosphate or glycerol were consistent with an active de novo synthesis of the monoenoic and dienoic phosphatidylinositols, followed by a deacylation-reacylation cycle for the introduction of arachidonate [276]. Marked preferences for arachidonoyl-CoA in the acylation of 1-acyl-*sn*-glycero-3-phosphoinositol have been reported for acyltransferases from rat liver [277] and brain [278].

An important role is ascribed also to deacylation-reacylation processes in the modulation of de novo-synthesized monoenoic and dienoic species of phosphatidylcholine in lung to provide the fully saturated, mainly dipalmitoyl, phosphatidylcholine species. The latter constitute the major component supporting pulmonary surfactant function. Phospholipase A_2 activities, necessary for the removal of monoenoic and dienoic acyl chains, have been reported in lung tissue [23,279] and a preference for unsaturated over saturated species was recently described [281]. The synthesis of disaturated phosphatidylcholine in lung tissue will not be discussed here further since some recent reviews are available [282–284].

VD. Bacterial phospholipases

The function of bacterial phospholipases, with emphasis on *E. coli* phospholipases, is less clear at the moment [301]. *E. coli* contains a well characterized phospholipase A_1 [17], located in the outer membrane [17,64]. This enzyme hydrolyzes both diacyl phosphoglycerides and 1-acyl lysophosphoglycerides [17,285] (compare subsection IIIA). Some phospholipase A_2 activity has been detected as well in the cell wall. The properties of the A_1 and A_2 activities in membranes [285,305] and those of a purified phospholipase from *E. coli* K-12 [62] strongly suggest that a single enzyme is responsible for the cleavage of acyl ester bonds at both positions. Genetic evidence is in line with this interpretation [303]. A less well characterized phospholipase A in the cytoplasm exhibits a preference for phosphatidylglycerol [293]. In addition to these enzymes, several lysophospholipases have been detected in *E. coli*. Notably, the inner membrane contained an enzyme which was twice as active towards 2-acyl lysophosphatidylethanolamine when compared to the 1-acyl isomer [285]. Using classical protein-purification techniques, a soluble lysophospholipase was purified about 1500-fold to near homogeneity by Doi and Nojima [286]. This enzyme hydrolyzed the 1-acyl isomer of lysophosphatidylethanolamine at a rate about 3-times faster than that measured for the 2-acyl isomer. It would thus seem that the inner membrane and cytosol fraction is equipped with enzymes to catabolize further lysophospholipids formed initially by either the phospholipase A_1 in the outer membrane or the cytoplasmic phospholipase. As noted above, the function of these lipolytic activities is still rather obscure. It has been repeatedly reported that no detectable turnover of the major *E. coli* phosphoglyceride, phosphatidylethanolamine, takes place in exponentially growing cells [287–290]. Turnover of phosphatidylglycerol and cardiolipin is easily measurable, but has been explained by conversion of phosphatidylglycerol into cardiolipin and of the polar headgroups of these phospholipids into a family of water-soluble oligosaccharides containing glycerophosphate moieties [291,292]. Phospholipases A have not been implicated in these conversions. These data would seem to exclude a role for the phospholipases A in normally growing cells.

In their original article on the purification of the membrane-bound phospholipase A_1, Scandella and Kornberg [17] have considered a number of other possible functions for this enzyme. Among these is that the enzyme may be responsible for phospholipid breakdown and subsequent changes in membrane integrity following phage infection. Indeed, when cells were infected with bacteriophage T4, radioactive lysophosphatidylethanolamine accumulated in parallel with loss of radioactivity from phosphatidylethanolamine. However, lysophospholipid production could equally well be elicited by osmotic lysis of cells in the absence of phage. While these experiments clearly show that the latent phospholipase can be activated to degrade the phospholipids in its own membrane, they also demonstrate that phage infection is not the only membrane-perturbing mechanism that leads to activation of the phospholipase. A variety of other treatments, including addition of colicins [294] and chemicals such as chloramphenicol, penicillin, toluene and formaldehyde [294], heat treatment [17,295] and spheroplast formation [295] have been shown to induce phospholipid hydrolysis in *E. coli*. Nevertheless, several authors have attempted to investigate whether phage-induced lysis is preceded by phospholipid hydrolysis. It is well known that lysozyme is produced after infection of *E. coli* with bacteriophage T4 and that mutants of the phage which lack the lysozyme gene fail to produce lysis of the host cell. It appeared an attractive hypothesis that membrane perturbation was required in order to permit lysozyme to reach the host peptidoglycan substrate

and that formation of free fatty acids and lysophospholipids by phospholipases constituted such a perturbation mechanism. Working along these lines, Cronan and Wulff [296,297] and Bradley and Astrachan [298] demonstrated that phospholipid hydrolysis can substantially precede lysis induced by infection with T4 phage or phage mutants. On the other hand, Josslin [299] arrived at the conclusion that phage infection did not necessarily enhance the rate of phospholipid hydrolysis detected in uninfected cells. Similar findings were reported by Bradley and Astrachan [298] when wild-type phage was used. It was concluded that phospholipid hydrolysis played no essential role in the phage life-cycle and that phospholipid hydrolysis was the consequence rather than the cause of the lysis process [298,299]. Such a conclusion has also been drawn with regard to the early effects of colicins. Increases in phospholipase A activity were found to occur well after colicin had caused changes in envelope structure and had exerted its complete effect on intracellular ATP and potassium levels [300].

A more direct approach applied by Doi et al. [293] to elucidate the function of *E. coli* phospholipases has largely confirmed these conclusions. On the basis of their initial studies, these authors [293] distinguished two phospholipase A activities in *E. coli*. A detergent-resistant phospholipase A (dr-phospholipase A), stable or even activated in the presence of detergents and organic solvents, was located in the particulate fraction and is now known to be present in the outer membrane. The enzyme is stable or activated by heat treatment at 60°C, requires Ca^{2+} and hydrolyzes both phosphatidylethanolamine and phosphatidylglycerol, preferentially at the *sn*-1-position. In contrast, a detergent-sensitive phospholipase A (ds-phospholipase A) hydrolyzing only phosphatidylglycerol was detected in the soluble fraction. This enzyme is inactivated by detergents, organic solvents and heat treatment at 60°C and its Ca^{2+} requirement is much less pronounced. The preferential position of attack could not be determined since no accumulation of lysophosphatidylglycerol accompanied the hydrolysis of phosphatidylglycerol [293]. By using an ingeneous selection technique, in which the product free fatty acid of the dr-phospholipase A was used to support growth of a fatty acid auxotroph of *E. coli*, mutants lacking either dr-phospholipase or both dr- and ds-phospholipase were isolated [302, 304]. A mutant deficient in only the ds-phospholipase has been constructed from the dr^-ds^--mutant by conjugation [306]. The availability of dr^--, ds^-- and dr^-ds^--mutants has greatly facilitated studies on the possible functions of *E. coli* phospholipases. Thus, Lusk and Park [307] were able to demonstrate that the dr^-ds^--mutant was as sensitive to colicin K as the dr^+ds^+-parent, despite the fact that colicin did not promote hydrolysis of phosphatidylethanolamine in the mutant. Both phage lambda [308] and phage T4 [308–311] induced normal cell lysis in the mutants, while no phospholipid hydrolysis was detectable. Although the use of these mutants has clearly established that phospholipid hydrolysis is not a prerequisite for colicin action or phage-induced lysis, studies with the mutants have thus far provided no hint as to the function of *E. coli* phospholipases. Doi and Nojima [306] have compared the growth characteristics, lipid composition and phospholipid turnover of the three mutants and the parent strain. No marked difference was detected in these parameters. It is relevant to note here that homogenates of the dr^-ds^--mutant, when assayed with aqueous dispersions of phosphatidylethanolamine, contained less than 1% of the parent-homogenate phospholipase A activity. However, autolysis of endogenous phospholipids in mutant homogenates amounted to about 5% of the values obtained with the parent strain. This could, perhaps, indicate that *E. coli* contains additional lipolytic enzymes, yet to be identified. This possibility was also raised by the studies of Nelson and Buller [311]. The authors found a phospholipase A associated with

T4 phage or T4 particles. This phage-associated phospholipase A was also found when the phage was grown in the dr^-ds^--mutant. Although this could mean that this phospholipase is phage gene specific, the alternative explanation could be that a hitherto undetected phospholipase in the dr^-ds^--mutant specifically adheres to the phage particle. The phage-associated phospholipase showed specificity for phosphatidylglycerol and in this respect resembled the ds-phospholipase of *E. coli*. It differed from this enzyme, however, in that it was activated rather than inhibited by methanol and Triton X-100. When extracts of the dr^-ds^--mutant were incubated with phosphatidylglycerol in the presence of 20% methanol, the residual phospholipase activity amounted to nearly 50% of the level found in dr^+ds^+-parent extracts. These data could be in line with the presence of other phospholipases in *E. coli* which were not previously detected due to the assay conditions.

Also, the possibility that the dr-phospholipase A, which is located in the outer membrane, would play a role in the digestion of phospholipids in the medium was not supported by experimental findings. No bacterial growth was observed in the presence of phospholipids as the sole carbon source [306]. What, if any, function the phospholipase A-catalyzed degradation of phospholipids has in the physiology of *E. coli* remains to be established.

VI. Concluding remarks

It is clear from the preceding discussion that phospholipid degradation by intracellular phospholipases A and lysophospholipases is an exceedingly complex phenomenon. Despite numerous studies on phospholipid catabolism, much remains to be learned in the future. Progress in science is preceded by asking questions. Many of the most elementary questions to be answered in future studies were put forward in the discussion of the results obtained so far and will not be repeated here. It is evident that some basic questions concerning subcellular distribution and functioning of particular phospholipases were treated superficially, if at all. For example, the effect of lysophospholipids on membrane structure and activity of membrane-bound enzymes was rarely discussed. In this regard, the reader is referred to a recent review in this series [312]. Nevertheless, it is hoped that the available evidence compiled in the foregoing pages will be of help in the design of experiments to unravel further the function and regulation of intracellular phospholipases. This undoubtedly will be accomplished by two lines of research. On the one hand, to understand the molecular basis of phospholipase action, it will be necessary to achieve solubilization of these enzymes from membranes followed by their complete purification with maintenance of enzymatic activity. Currently, in a limited number of cases, this goal has been reached (Section III). On the other hand, continued research is necessary on the factors which govern enzymatic activity of phospholipases in their natural environment, especially in membrane-bound form. Hopefully, in the near future, the gap between these two approaches can be bridged by the development of reconstituted systems of purified enzymes in model membranes. The availability of such systems will allow a systematic approach to the study of the physico-chemical factors which govern the action of membrane-bound phospholipases.

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