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Determination of the Catalytic Activity of Phospholipase A_2: *E. coli*-Based Assay Compared to a Photometric Micelle Assay

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Dedicated to Prof. Dr. R. Kattermann on the occasion of his 60th birthday

Summary: Phospholipase A_2 activity in human sera was determined of the basis of the *E. coli* assay and compared to a photometric micelle assay. The *E. coli* assay is based on the hydrolysis of phospholipids from [1-^{14}C]oleic acid-labelled *E. coli* biomembranes. In the photometric assay the phospholipase A_2 acts on mixed phospholipid micelles. The amount of fatty acid produced is quantitated in a subsequent photometric assay by coupling in the reaction to the coenzyme A metabolism. The *E. coli* membranes are essentially resistent to other lipases in human sera, i. e. lipoprotein lipases, hepatic triacylglycerolipase or pancreatic lipase and thus a very specific substrate for the phospholipase A_2 of human serum. The photometric assay, though, is susceptible to other lipases in human serum. The ratio of [1-^{14}C]oleic acid to released total fatty acids served as the basis for the calculation of the true enzymatic activity. The assay closely correlated with the photometric assay based on mixed micelles in the higher ranges of phospholipase A_2 activity, but not in the normal range. The sensitivity is higher by at least two powers of 10. The human serum phospholipase A_2 strongly preferred *E. coli* membranes as substrate to the mixed micelles containing phosphatidylcholine/phosphatidylethanolamine. In conclusion, the modified phospholipase A_2 assay based on *E. coli* membranes is a sensitive, specific, reliable, and convenient method for the measurement of phospholipase A_2 activity in human sera. The photometric assay suffers from low sensitivity but has the advantage of practicability in a normal routine laboratory, including the amenability to automation.

Introduction

Various efforts have been made to develop sensitive assays for the determination of phospholipase A_2[1]) (1–16). The choice of a detection method depends partly on the goal of a particular experiment. For example, some assays can be used on purified enzymes but are incompatible with crude systems, some methods provide a continuous assay and generate a time course while others do not, and some methods are amenable to automation while others are not. However, the most important consideration in the choice of the detection method is the sensitivity required for the particular enzyme. The required sensitivity depends on the quantity of enzyme available and on its specific activity. This point is especially important for the assay of non-pancreatic phospholipases A_2 in human plasma, which are found in lower quantities and are, in general, less active than their counterparts from the pancreas or venom.

The sensitivity of an assay is influenced by a number of factors, the most important of which is the detection limit of a particular method. The detection limit

[1]) Enzymes
Phospholipase A_2 = phosphatide 2-acylhydrolase (EC 3.1.1.4)
Phospholipase A_1 = phosphatide 1-acylhydrolase (EC 3.1.1.32)
Lipase = triacylglycerol acylhydrolase (EC 3.1.1.3)

is determined by physical properties of the assay method itself, such as the absorbance coefficient of a chromophore or the physical limitations of the instrumentation. In addition to the detection limit, the sensitivity of an assay depends on the turnover rate of the particular enzyme, the rate of background reaction, and, in some cases, on the assay time and volume.

Some assays are based on artificial substrates such as liposomes or micelles, which are possibly not the best substrates for the phospholipase A_2 in human plasma. Synovial phospholipase A_2, identical with serum phospholipase A_2, prefers membrane phospholipids as a substrate (17, 18). There is some evidence that membranes of *Gram*-negative bacteria are the natural substrate of several extracellular phospholipases (19).

Other assays use membranes labelled only with one radioactive tracer, e. g. oleic acid. They do not monitor the "true activity" of phospholipase A_2 because the released but unlabelled fatty acids are not taken into account. Therefore, it is not possible to calculate the phospholipase A_2 activity in U/l. Furthermore, there is evidence that the phospholipase A_2 activity depends on the fatty acid composition in the *sn*-2 position of the phospholipid (20).

The photometric micelle assay developed by *G. E. Hoffmann* (21), which has recently become commercially available, suffers from lack of sensitivity for normal and moderately elevated phospholipase A_2 activity concentrations as is shown below. But it monitors the true activity.

The *E. coli* membrane assay in the form described represents a method that combines the advantage of the sensitivity of a radiometric assay based on a natural substrate, with the determination of the true and total activity of phospholipase A_2. For this purpose the released radioactivity has been related to the sum of all liberated fatty acids. The principle of this method is the hydrolysis of radioactive oleate in the *sn*-2 position of membrane phospholipids. For the quantitation of the enzyme activity, the liberated oleate has to be separated from the remaining radioactive phospholipids. A common standard method for the separation of the lipids is silica gel thin-layer chromatography following extraction from the incubation mixture. This procedure is rather time-consuming. In contrast, the modified *E. coli* method allows a remarkable spreading of the test range and a reliable measurement of phospholipase A_2 activity in the early stages of sickness. We compared this method with the photometric assay (21).

Methods

E. coli-based phospholipase A_2 assay

Phospholipase A_2 (EC 3.1.1.4) activity was determined by using [1-^{14}C]oleate-labelled *Escherichia coli* (K12) as substrate with modifications first described by *Elsbach* (23). The essentials of the assay have been described earlier (24, 25). An *E. coli* culture was incubated with $7.4 \cdot 10^6$ Bq/l oleic acid bound to 0.4 g/l fatty acid free bovine serum albumin for 75 min at 37 °C. Thereafter, the bacteria were sedimented, resuspended in fresh medium, and allowed to incorporate the rest of the labelled precursor. The labelled bacteria were washed once with 10 g/l albumin in saline followed by repeated washings with saline. After centrifugation the *E. coli* were autoclaved for 15 min at 120 °C. This procedure inactivates bacterial phospholipases and renders the envelope phospholipids readily accessible to the action of serum phospholipase A_2. Prior to use, the *E. coli* suspension was lyophilized in appropriate aliquots in extraction tubes. More than 95% of the incorporated label was found in phospholipids and, as demonstrated by bee venom phospholipase A_2 hydrolysis, more than 95% of the [1-^{14}C]oleate was in the *sn*-2 position, as was proven by thin-layer chromatography (25).

The lyophilized labelled *E. coli* membranes (18 nmol phospholipid, 180 000 disintegrations per minute) were resuspended in an assay mixture containing 100 mmol/l Tris-HCl (pH 8.0), 5 mmol/l $CaCl_2$ and 20 g/l fatty acid free bovine serum albumin, and 25 µl of serum or other fluids, for a final volume of 150 µl. Reaction mixtures were incubated at 37 °C for 15 min while gently shaking to prevent the sedimention of the membranes. The reaction was stopped by addition of 100 µl of 30 mmol/l EDTA, and the lipids extracted with 1250 µl of modified *Dole* (26) reagent (propane-2-ol/*n*-heptane/1 mol/l H_2SO_4, 40 + 10 + 1, by vol.) followed by 200 µl of heptane and 750 µl of H_2O. After phase separation 0.5 ml of the organic phase

Tab. 1. Comparison of the procedures of the two evaluated phospholipase A_2 assays: radiometric *E. coli* assay and the photometric mixed micelle assay.

	Radiometric *E. coli* assay	Photometric micelle assay
Substrate	Autoclaved membranes, phosphatidylethanolamine : cardiolipin [80 : 20]	Mixed micelles, phosphatidylcholine : phosphatidylethanolamine [50 : 50], Triton-X, bile acids
Incubation	5 mmol/l Ca^{2+}, 37 °C, 15 min in the presence of albumin (2%)	3 mmol/l Ca^{2+}, 37 °C, 15 min
Reaction stop	EDTA (10 mmol/l)	EDTA (8 mmol/l)
Detection procedure	Radioactive free fatty acids: – *Dole*'s extraction – Solid-phase extraction – Scintillation counting	Increase in free fatty acids: – Enzymic determination of free fatty acids – Photometry

was passed through a reusable column filled with 100 mg of aminopropyl solid phase (Macherey & Nagel, Aachen, Germany). Free [1-C^{14}]oleic acid was quantitatively eluted with diethylether/acetic acid (2%) into a scintillation vial, while unhydrolyzed substrate remained on the column. The eluates were counted for 3 minutes (Scintillation cocktail: Quickzint, Zinsser-Analytic, England, Scintillation counter: LKB 1215 Rackbeta). Blank activity, resulting from residual [1-^{14}C]oleic acid not incorporated into lipids, was substracted for each sample. Enzyme activity was calculated as released fatty acids based on the ratio between counted radioactivity and total liberated fatty acids as determined by HPLC (27, 28), if not otherwise indicated. The fatty acid pattern and the relative concentrations for serum phospholipase A_2 were determined with purified human serum phospholipase A_2 as a standard, in contrast to l.c. (25). The phospholipase A_2 was isolated and purified from sera of intensive-care patients [manuscript in preparation]. 1 U/l was equivalent to 4.8×10^6 disintegrations per minute and litre.

Micelle phospholipase A_2 assay

The activity of phospholipase A_2 was determined by the photometric assay recently described by *Hoffmann* et al. (21). Briefly, a lyophilized phospholipid/detergent mixture containing phosphatidylcholine, phosphatidylethanolamine (mass fraction 0.5), Triton X-100 and sodium deoxycholate (Boehringer Mannheim, Germany) was used throughout. The total liberated free fatty acids were determined with an enzymatic test kit (Boehringer Mannheim, Germany) based on a coenzyme A reaction. Fatty acids are incorporated into acyl-CoA using acyl-CoA synthetase, then oxidized to 2,3-*trans*-enoyl-CoA with acyl-CoA oxidase. The peroxide generated in the latter step leads to the formation of a chromophore by oxidative coupling of an aminoantipyrine and a phenol derivative. Alternatively, the fatty acids were quantitated separately by the HPLC method (27, 28). For direct comparison, the *E. coli* assay and the photometric micelle assay are described in table 1.

Acute phase proteins

The acute phase proteins, C-reactive protein and serum amyloid A, were determined by nephelometry (Behring, Marburg, Germany). The antiserum against serum amyloid A was a friendly gift of the Behring company.

Results

Character of the substrate

We investigated the *E. coli* assay for interference from various lipases. Human serum contains several lipases and phospholipases, i.e. lipoprotein lipase, hepatic triacylglycerolipase, pancreatic lipase, which might influence the accuracy of the assay. Therefore, *E. coli* membranes were incubated with different (phospho)lipases. The phospholipids of *E. coli* membranes were either labelled with [1-^{14}C]palmitic acid in the *sn*-1 position or with [1-^{14}C]oleic acid in the *sn*-2 position (tab. 2). Control labelling of the *sn*-1 position was insofar important as the radioactive oleic acid was also incorporated into the *sn*-1 position (5 to 10 percent), and high activity of phospholipase A_1 might simulate phospholipase A_2 activity. To check the effects of serum lipoprotein lipases the *E. coli* membranes were incubated with sera from healthy volunteers after a bolus injection of heparin (5000 i.U., i.v.) collected after 10 min. Those sera are rich in lipoprotein lipase and hepatic triacylglycerolipase.

Tab. 2. The specificity of the *E. coli* membranes as substrate for the serum phospholipase A_2 activity was verified by incubation with different (phospho)lipases. For this purpose, the phospholipids of *E. coli* membranes were labelled either with [1-^{14}C]palmitic acid in the *sn*-1 position or with [1-^{14}C]oleic acid in the *sn*-2 position to the ratio indicated in the table. To check the activity of serum lipases on the *E. coli* membrane healthy volunteers (n = 3) were heparinized with 5000 i.U. of heparin and blood was drawn after 10 min. Such sera are rich in lipoprotein lipases and hepatic triacylglycerolipase. The latter exhibits mainly phospholipase A_1 activity. Sera of intensive-care patients (n = 3) with nearly the same activity on the micellar substrate were chosen for comparison. Additionally, pancreatic lipase was added to a normal serum and tested for the release of radioactive fatty acids from the *E. coli* membranes. The sera of healthy volunteers (n = 3) served as controls.

	Release of fatty acids from the phospholipids		
	Micelle assay	*Escherichia coli*-based assay	
	Total fatty acids µmol/l/min	[1-^{14}C]Oleic acid nmol/l/min	[1-^{14}C]Palmitic acid nmol/l/min
Sera of healthy volunteers before heparin (n = 3)	0.05 ± 0.01*	5.1 ± 0.2	0.7 ± 0.2
Sera of healthy volunteers 10 min after heparin 5.000 I.E. (n = 3)	20.4 ± 1.0	5.7 ± 0.3	3.6 ± 0.4
Phospholipase A_2-rich sera of intensive-care patients (n = 3)	20.0 ± 1.5	1360 ± 120	270 ± 21
Normal sera enriched with pancreatic lipase [1000 U/l] without colipase (n = 3)		5.3 ± 0.4	6.4 ± 0.5
Labelling ratio of *sn*-2 and *sn*-1 position		95 : 5	20 : 80

* 24 h incubation

The latter exhibits mainly phospholipase A_1 activity. Its activity on the micelle substrate was about 20 µmol/min · l. The release of the labelled oleic acids was on the same order of magnitude as in the sera of healthy volunteers without heparin application, and somewhat higher for the labelled palmitic acid. This demonstrates a considerable resistence of the *E. coli* membranes to the activities of phospholipase A_1 and the lipoprotein lipase. Control experiments were done with phospholipase A_2-rich sera from intensive-care patients. The sera were diluted to approximately 20 U/l (332 nkat/l) as determined by the photometric micellar assay. Radioactive oleic acid was released from the *sn*-2 position, as expected. The liberation of labelled palmitic acid can be explained by the co-labelling of the *sn*-2 position. This effect reflects exactly the ratio of the label between the *sn*-1 and *sn*-2 position. Acute pancreatitis often proceeds with an increase in phospholipase A_2, and it is important to distinguish this activity from that of the pancreatic lipase. Therefore, we incubated the labelled membranes with normal serum spiked with pancreatic lipase to 1000 U/l (16667 nkat/l). As shown in table 2 the release of labelled fatty acids is barely different from that of normal sera.

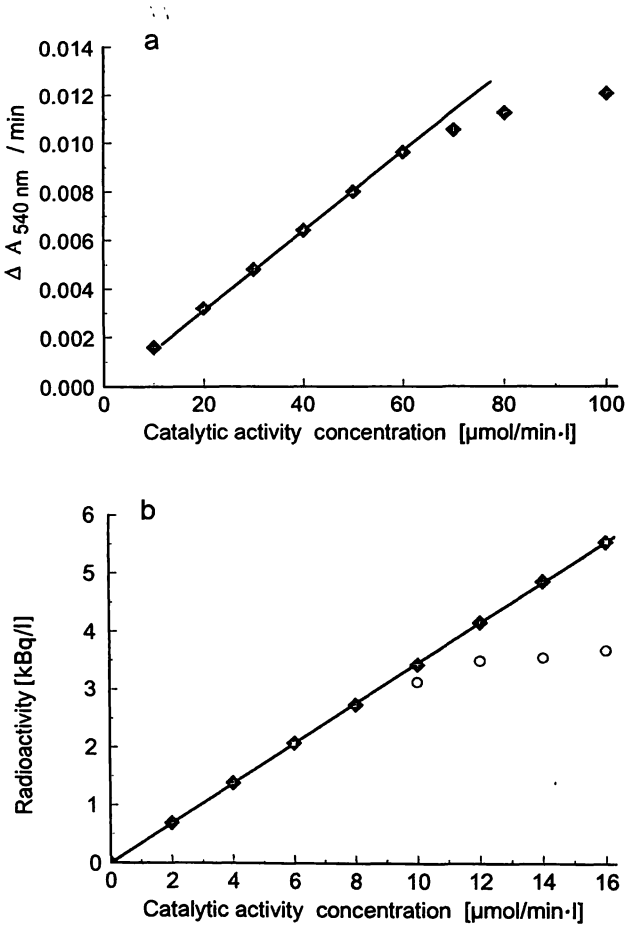

Fig. 1. Linearity of the phospholipase A_2 assays.
a) The linear range for serum phospholipase A_2 was established by serial dilution ◆. The cut-off value for the photometric micelle assay was 58 U/l (967 nkat/l).
b) Using the *E. coli* assay the linear range was established in the same manner o. The cut-off value for the radiometric *E. coli* assay of human serum was 7.2 µmol/min · l (120 nkat/l).
Additionally, the linear range was determined with purified phospholipase A_2 isolated from human sera with high phospholipase A_2 contents ◆. The activity of purified human serum phospholipase A_2 was found to be linear up to 14.4 U/l (240 nkat/l).

Analytical properties

Linearity

Using sera from septic patients the linear range for serum phospholipase A_2 activity was established up to 7.2 U/l (120 nkat/l) (fig. 1) corresponding to a hydrolysis rate of 8%. Under assay conditions the curve deflected from linearity at 42 × 10^6 disintegrations per minute and litre. To monitor the enzyme activity beyond this range, the sera of patients with septicaemia and polytraumatic shock must be diluted many times. Surprisingly, the activity of purified human serum phospholipase A_2 was found to be linear up to a hydrolysis rate of 16%, corresponding to 14.4 U/l (240 nkat/l). Spontaneous hydrolysis of membrane phospholipids was not observed. The range of the photometric micelle assay was found to be linear up to 60 U/l (1000 nkat/l) (21).

Reliability

Quality control data for intra-assay and inter-assay imprecision for the *E. coli* and the photometric micelle assay, resp. are given in table 3.

Tab. 3. Inter-assay imprecision of the radiometric phospholipase A_2 assay with *E. coli* membranes and the photometric micelle assay [U/l].

Radiometric *E. coli* assay					
Intra-assay imprecision[a]			Inter-assay imprecision[b]		
Mean [U/l]	SD [U/l]	CV (%)	Mean [U/l]	SD [U/l]	CV (%)
0.32	0.018	5.8	0.65	0.063	9.7
3.1	0.11	3.5	3.3	0.21	6.3
7.7	0.22	2.9	7.1	0.38	5.4
Photometric micelle assay					
Intra-assay imprecision[a]			Inter-assay imprecision[b]		
Mean [U/l]	SD [U/l]	CV (%)	Mean [U/l]	SD [U/l]	CV (%)
17.8	1.58	8.9	16.8	1.62	9.6
40.7	1.42	3.5	42.5	2.83	6.6

[a] Intra-assay imprecision was determined with three different serum pools (n = 12 in each case).
[b] Inter-assay imprecision was determined with three other different serum pools (n = 20 in each case)

Sensitivity

The limit of detection for the photometric micelle assay was established using various concentrations of fatty acids in samples free of phospholipase A_2 activity. This was done in contrast to *Hoffmann* et al. (21) who verified the detection limit using water instead of serum. The detection limit depended on the fatty acid concentration and increased from 5.1 U/l (85 nkat/l) (without fatty acids in the sample) to 12.7 U/l (212 nkat/l) (fatty acid concentration 2150 µmol/l in the sample) (fig. 2). The radiometric *E. coli* assay was almost independent of the fatty acid concentration, and the detection limit was 0.35 U/l (5.8 nkat/l) (fig. 2). It has to be mentioned that the activities obtained by both assays are not exactly comparable because the substrates for the assays are different.

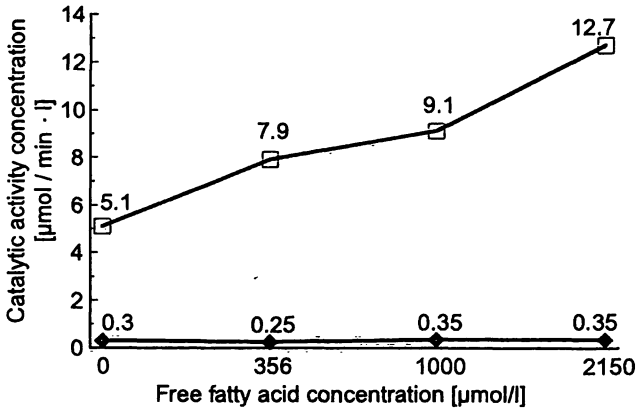

Fig. 2. Detection limits for the *E. coli* assay (◆—◆) and the photometric micelle assay (□—□) were established using various concentrations of fatty acids in the sample without phospholipase A_2 activity (abscissa). The detection limits of each point is given as mean + 3 s (standard deviations).

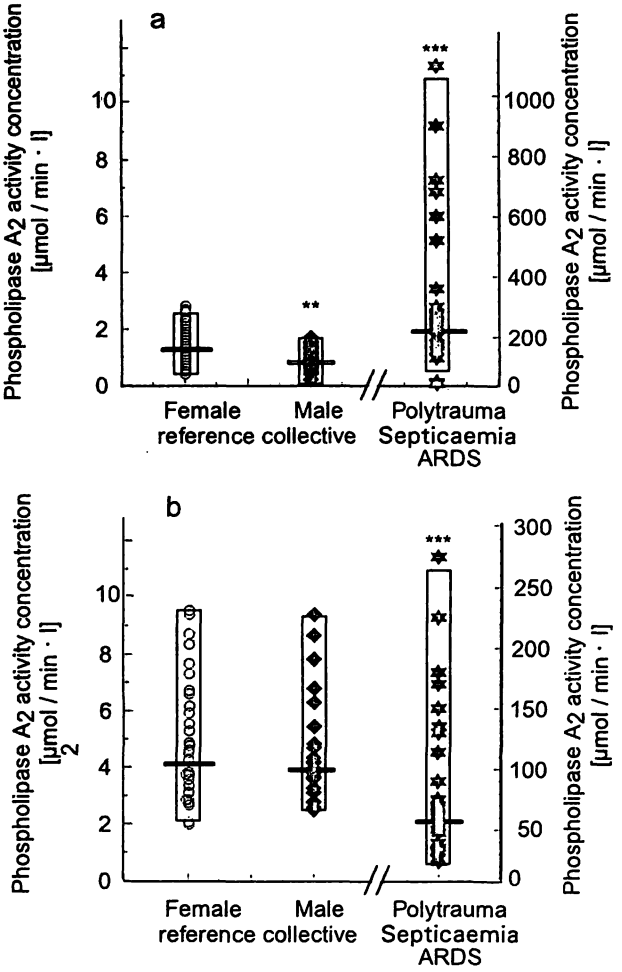

Fig. 3. a) Phospholipase A_2 activity in sera from healthy male (n = 43) and female volunteers (n = 55) and intensive-care patients (n = 36). The boxes show the median and the 2.5 to 97.5 percentile range of the results. In the *Mann-Whitney* two sample test the differences between the groups were statistically significant (** $p < 0.01$, *** $p < 0.001$),
b) Using the photometric assay the reference ranges were established with the same specimens. No statistical difference could be observed between the male and female groups.

Reference range and patients

Sera from healthy volunteers and intensive-care patients were analyzed with the *E. coli* method (fig. 3a) and compared to the photometric assay. Reference ranges (2.5 to 97.5 percentile) were established in healthy women (n = 45) as 0.088—2.22 U/l (1.46—36.8 nkat/l) and in healthy men (n = 40) as lower than 0.88 U/l (14.66 nkat/l). In the unpaired non-parametric test (*Mann-Whitney*) the medians of healthy males and females were significantly different ($p < 0.001$). This is the first observation of sex-specific reference ranges for serum phospholipase A_2, but at present nothing can be said about this difference or its clinical implications. In addition, patients with pathologically high phospholipase A_2 activities were investigated to demonstrate the applicability of this assay. Sera of intensive-care patients (n = 36) suffering from septicaemia, pancreatitis, acute lung failure and other diseases reached values up to 1080 U/l (18 000 nkat/l).

For the photometric assay, we plotted the phospholipase A values of the same healthy volunteers (fig. 3b). The seeming reference range extended from 2 to 9 U/l (2.5 to 97.5 percentile). The free fatty acid concentrations of all volunteers were below 700 µmol/l. No sex-specific differences could be observed. The sera of the patients of the intensive-care unit reached values up to 290 U/l (4865 nkat/l). Actually, the reference range must be estimated with the sensitivity range corresponding to the actual fatty acid concentration in the specimen, i.e. probably below 7.9 U/l (132 nkat/l) for low fatty acid concentration and below 12.7 U/l (212 nkat/l) for high fatty acid concentration. Values below 7.9 U/l (132 nkat/l) have to be considered as accidental.

Correlation of the *E. coli* assay with the micelle assay

The photometric micelle assay lacks analytical sensitivity in the normal and moderately elevated phospholipase A_2 range. For this reason we compared both methods at higher phospholipase A_2 ranges only. The results obtained are plotted in figure 4. They demonstrate an unexpectedly high correlation ($r = 0.97$, $p < 0.001$). However, the slope (4.1) indicates the obvious preference of phospholipase A_2 for the membraneous substrate. This may partly be caused by the substrate composition, implying that phosphatidylethanolamine is possibly preferred over other phospholipids e. g. phosphatidylcholine. The micellar substrate contains phosphatidylcholine and phosphatidylethanolamine in a mass ratio of 1 : 1, since phosphatidylcholine was not hydrolized and is thus not a suitable substrate for phospholipase A_2 (29). The phospholipase A_2 activity, though, is largely dependent on the composition and conformation of the substrate (30).

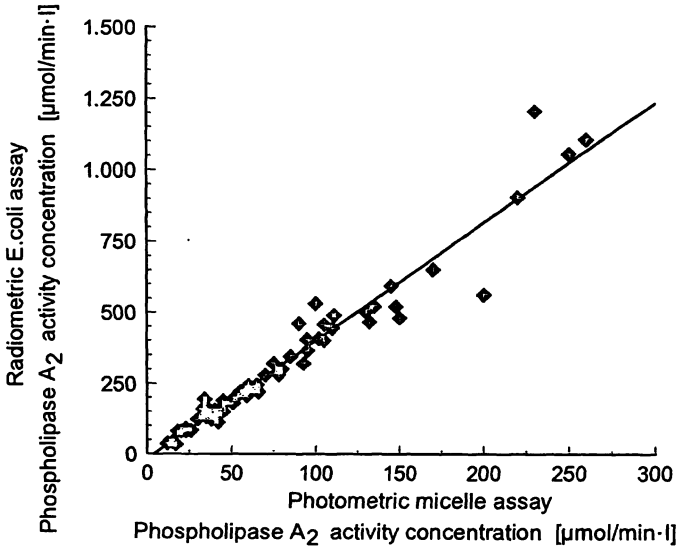

Fig. 4. Comparison of the *E. coli*-based radiometric assay and the micelle assay for the determination of phospholipase A_2 activity in human sera. The micelle assay showed markedly lower activity than the membrane assay. $R = 0.97$; $y = 4.1x - 15.3$; $N = 55$. For comparison, only phospholipase A_2 activity above 15 U/l was taken because of the detection limit of the photometric assay.

Comparison to other acute phase proteins

Figure 5 demonstrates a typical time course of acute phase proteins of a polytraumatized patient from the time of admission. The kinetics of C-reactive protein, serum amyloid A, and phospholipase A_2 measured by the *E. coli* assay paralleled each other well in the initial phase, whereas the phospholipase A_2 determined by the photometric micelle assay shows seeming lag phase. The phospholipase A_2 determined by the photometric assay showed a significant increase only 24 to 32 h after the event, whereas the *E. coli* assay reacted with a ten-fold increase after 8 h, similar to the kinetics of C-reactive protein and serum amyloid A.

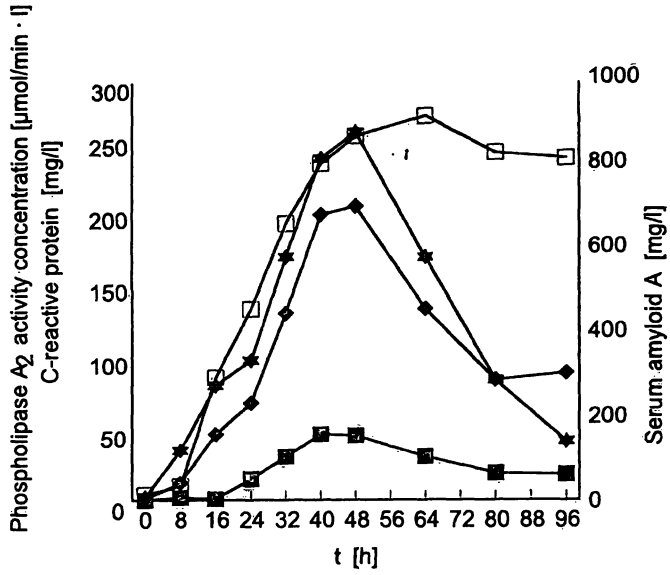

Fig. 5. Typical time course of acute phase proteins of a polytraumatized patient beginning with time of admission:
- ■ phospholipase A_2 (photometric micelle assay);
- ◆ phospholipase A_2 (radiometric *E. coli* assay);
- ✳ serum amyloid A;
- □ C-reactive protein.

Discussion

The selection of a membrane fatty acid labelled phospholipid substrate from *E. coli* allows an approximation of the physiological array of phospholipids and may avoid inherent problems associated with the physicochemical characteristics of synthetic substrates. The disadvantages are that the phospholipid composition is controlled by *E. coli* and can only be controlled with differently, using highly sophisticated growth conditions. Secondly, the amount of phospholipid substrate present in the assay is extremely low. On the other hand, the characteristics of phospholipid micelles, i. e. packing density and lipid surface charge, can also have marked effects on phospholipase A_2 activation and hydrolysis (8, 30). These circumstances make a standardization of a catalytic assay quite difficult.

This work indicates that phospholipase A_2 activity in human serum hydrolyzed phosphatidylcholine/phosphatidylethanolamine/deoxycholic acid/Triton X-100 mixed micelles poorly, and that autoclaved *E. coli* membranes containing primarily radio-labelled phosphatidylethanolamine were readily hydrolyzed by the

enzyme, thus providing the basis for a sensitive assay system. Similar results were described by *Parks* et al. (31). There is no preference of any acyl chain in the *sn*-2 position (33), in contrast to phospholipases A_2 from platelets and rheumatoid synovial fluid (2, 20).

Another problem is inherent to the micellar photometric assay, which is based on the determination of total fatty acids including those present in the serum of the patient as so-called free fatty acids before the phospholipase A_2 assay. The sera of intensive-care patients often contain high free fatty acid concentrations and the additionally liberated fatty acids from the phospholipase A_2 activity contribute little to an increase in the total free fatty acids (e. g. corresponds the free fatty acid concentration of about 2500 µmol/l, not uncommon in sera of intensive-care patients, to an absorbance of 0.417). A phospholipase activity of 8 U/l (133 nkat/l) causes an increase in absorbance of 0.020 over a period of 15 min (0.0013/min). The increase amounts to less than 5%. The multiple steps of the assay (21), which are impeded often by a lack of automation, imply that the increase in the phospholipase activity can not reliably be distinguished from the basal free fatty acid concentration, since low differences of absorbance lie within the sensitivity range. This explains why it is not possible to determine phospholipase A_2 activity below 8 U/l (133 nkat/l) by the photometric micelle assay with an acceptable accuracy. In comparison, the upper limit of the linear range of the *E. coli* assay was 7.2 U/l (120 nkat/l) and the assay shows good spreading in this range.

The preference for *E. coli* membranes as substrate suggests that the enzyme may function in the host defence against *Gram*-negative bacteria (19). Assuming that the polymorphonuclear leukocytes represent one possible source of serum phospholipase A_2 (7), serum phospholipase A_2 may be highly specific for *Gram*-negative membranes, and even more so after activation of phospholipase A_2 by the bactericidal/permeability-increasing protein (32).

It was possible to demonstrate that the *E. coli* membrane assay was very specific for the phospholipase A_2 of human serum and relatively insensitive to other lipases in human sera, i. e. lipoprotein lipases, hepatic triacylglycerolipase or pancreatic lipase. Although the activity of phospholipase A_1 (hepatic triacylglycerolipase) from human serum on *E. coli* membrane phospholipids appears to be very low in terms of cleaved, labelled fatty acids, the hydrolysis of the *sn*-1 fatty acids resulted in a slight increase in radioactive lysophosphatidylethanolamine as assessed by the release of [^{14}C]palmitic acid (see tab. 2: 0.7 vs. 3.6 nmol/min · l). Only 5% to 10% of the fatty acid label is incorporated into the *sn*-1 position, and lyso-phosphatidylethanolamine is retained by the solid-phase column technique for the separation of fatty acids. Consequently, it does not contribute to the result.

In comparison, the photometric micelle assay is not affected by the phospholipase A_1 activity of the specimen, as we have recently demonstrated (33). The influence of therapeutic heparinization on phospholipase A activity was investigated in healthy volunteers who received an intravenous bolus injection of 5000 i. U. of heparin. Total phospholipase A activity 10 min after injection increased up to 22 U/l and dropped to < 5 U/l after several hours.

Activity was expressed accounting for the sum of the released fatty acids. In previous publications (29, 34) the phospholipase A_2 activity of the radiometric *E. coli* assay was expressed as the hydrolysis rate based only on the rate of release of the labelled fatty acids. This renders comparison and longitudinal studies questionable because of the possible variability in the degree of specific labelling from lot to lot. A recent paper focussing on the comparison of the radiometric assay and the photometric assay dealt only with the correlation of the patients' results (35). The authors obtained a similar high correlation of the two methods. In addition, the concordance of each pair of values was extremely good even on the basis of the measured activity. But the authors did not comment on the determination and calibration of the true activity of the enzyme on the *E. coli* membranes. We were able to demonstrate an obvious preference of the enzyme for the membranous substrate (fig. 4) resulting in activity four times as high. This, in turn, contributes additionally to the higher sensitivity of the biomembrane assay.

The relatively small linear range of serum phospholipase A_2 compared to purified phospholipase A_2 from human plasma might be explained by the presence of an inhibitor. Besides lipocortins, lipoproteins may play an important role in the mechanism of inhibiton (36). The same phenomenon may also be responsible for the relatively low activity of serum phospholipase A_2 on mixed micelles where the serum fraction is markedly higher.

The concordant kinetics of phospholipase A_2 activity and the other acute phase proteins (serum amyloid A and C-reactive protein) in the time-course study are compatible with the conclusion that phospholipase A_2 in human plasma has its origin in the hepatocytes, and can be considered as an acute phase protein itself. This hypothesis has been supported by *Crowl* et al. (17) who were able to demonstrate a common genetic regulation of phospholipase A_2 and of the above-

mentioned acute phase proteins in HepG2 cells. And indeed, we very recently were able to prove the expression and secretion of phospholipase A_2 in primary human hepatocytes (data not yet published).

In conclusion, the *E. coli*-based assay is more reliable and more sensitive than the photometric micelle assay for the determination of the specific activity of phospholipase A_2 in human serum. The membrane substrate is easily prepared. The reference range is below 2.22 U/l (37 nkat/l). With the method described the activities of phospholipase A_2 of human serum samples can be reliably determined. The inter- and intraassay imprecisions were below 5% and 10%, resp. Pathologically elevated phospholipase A_2 activity increased up to 500-fold above the reference range, with indicates a high diagnostic potential.

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