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# Sphingosine Kinase in Blood Platelets

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**Summary:** In a previous communication the enzymatic phosphorylation of the long chain bases sphinganine (dihydrosphingosine), 4*t*-sphingenine (sphingosine), 4*D*-hydroxysphinganine (phytosphingosine) and 3-dehydrosphinganine (3-ketodihydrosphingosine) with erythrocyte preparations from human and rabbit blood has been described. Studies with purified human and pig thrombocytes revealed a highly active ATP-dependent kinase in this blood cell. A reinvestigation of the sphingosine kinase activity in erythrocytes prepared by our previous procedure demonstrated that the kinase activity is proportional to the number of platelets, but that erythrocytes are devoid of this enzyme.
A simple quantitative radio-assay for the phosphate esters, which are rather insoluble in water and organic solvents, has been worked out.

*Sphingosin-Kinase in Thrombozyten*

**Zusammenfassung:** In einer früheren Mitteilung beschrieben wir die enzymatische Phosphorylierung der langkettigen Basen Sphinganin (Dihydrosphingosin), 4*t*-Sphingenin (Sphingosin), 4*D*-Hydroxysphinganin (Phytosphingosin) und 3-Dehydrosphinganin (3-Ketodihydrosphingosin) mit Erythrozyten-Präparationen von Menschen- und Kaninchenblut. Untersuchungen mit gereinigten Menschen- und Schweinethrombozyten ergaben nun, daß diese eine hoch wirksame ATP-abhängige Kinase enthalten. Eine erneute Untersuchung der Sphingosin-Kinase-Aktivität in den Erythrozyten-Präparationen ergab, daß die Kinase-Aktivität proportional zur Thrombozytenzahl der Erythrozyten-Präparation ist. Erythrozyten sind im wesentlichen frei von Kinase-Aktivität. Ein einfacher quantitativer Radioaktivitätstest zur Bestimmung der Phosphatester der langkettigen Basen, die sich als sehr unlöslich in Wasser und organischen Lösungsmitteln erweisen, wird beschrieben.

In a previous communication[1] we described the enzymatic phosphorylation of the long chain bases sphinganine (dihydrosphingosine), 4*t*-sphingenine (sphingosine), 4*D*-hydroxysphinganine (phytosphingosine) and 3-dehydrosphinganine (3-ketodihydrosphingosine) with human and rabbit erythrocyte preparations. During our further search for metabolic reactions of sphingolipids in corpuscular elements of the blood we observed the very efficient phosphorylation of 4*t*-sphingenine and sphinganine by human and pig thrombocytes. In order to decide whether erythrocytes or thrombocytes possess the long chain base kinase, we developed a method for the purification of platelets and erythrocytes. In addition a quantitative radioassay for the determination ot the phosphorylated sphingosine bases, which are rather insoluble in polar and non-polar solvents, has been developed. This procedure, which proved highly reproducible and simple, allowed the assay of kinase activity in red blood cells and platelets. In this paper it will be shown that only platelets contain a highly active kinase, which is a soluble enzyme of the cytosol fraction. Our previous erythrocyte preparations contained enough thrombocytes to explain the good yields of sphingosine phosphate esters. Erythrocytes are essentially free of sphinganine kinase.

*Enzyme:*

Sphingosine kinase, ATP:sphingosine 1-phosphotransferase (EC 2.7.1.?, not yet listed). Sphingosine stands for all naturally occurring long chain bases.

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[1] Stoffel, W., Assmann, G. & Binczek, E. (1970) *this J.* **351**, 635–642.

## Results and Discussion

The procedures for the purification of human and pig platelets and erythrocytes described under Experimental yielded one platelet per $1.5 \times 10^3$ erythrocytes and less than 1 erythrocyte per $10^4$ platelets. The platelet preparation should be sufficiently pure to obtain unambiguous results regarding the question whether the sphingosine kinase is present in platelets or erythrocytes particularly if labelled substrates such as [3-$^{14}$C] sphinganine and [3-$^{3}$H]sphingenine are used.

When whole thrombocytes (Fig. 1a) and erythrocytes (Fig. 1b) were incubated with these substrates in independent experiments, it became quite clear that only platelets possess the kinase activity, as seen in Fig. 1 in which the two labelled compounds were sphinganine 1-phosphate (A) and unreacted substrate (B). The incubation with erythrocytes yielded no phosphate ester. In another experiment, shown in Fig. 2, the number of platelets was correlated with the yield of 1-phosphate ester formed. The relationship was linear. When equal numbers of erythrocytes are incubated under identical conditions, only trace amounts of product are formed and no relationship between number of erythrocytes and enzymatic activity is obvious. This proves that only platelets contain the enzymatic activity. The result indicates that the erythrocyte fraction used in our previous investigation, which was prepared by a different technique, did contain an appreciable number of platelets. In fact if we sedimented the erythrocytes at $650 \times g$ for 30 min, as described before, all platelets sedimented too. Preparations like these yielded 30% product.

We further localized the kinase activity within the

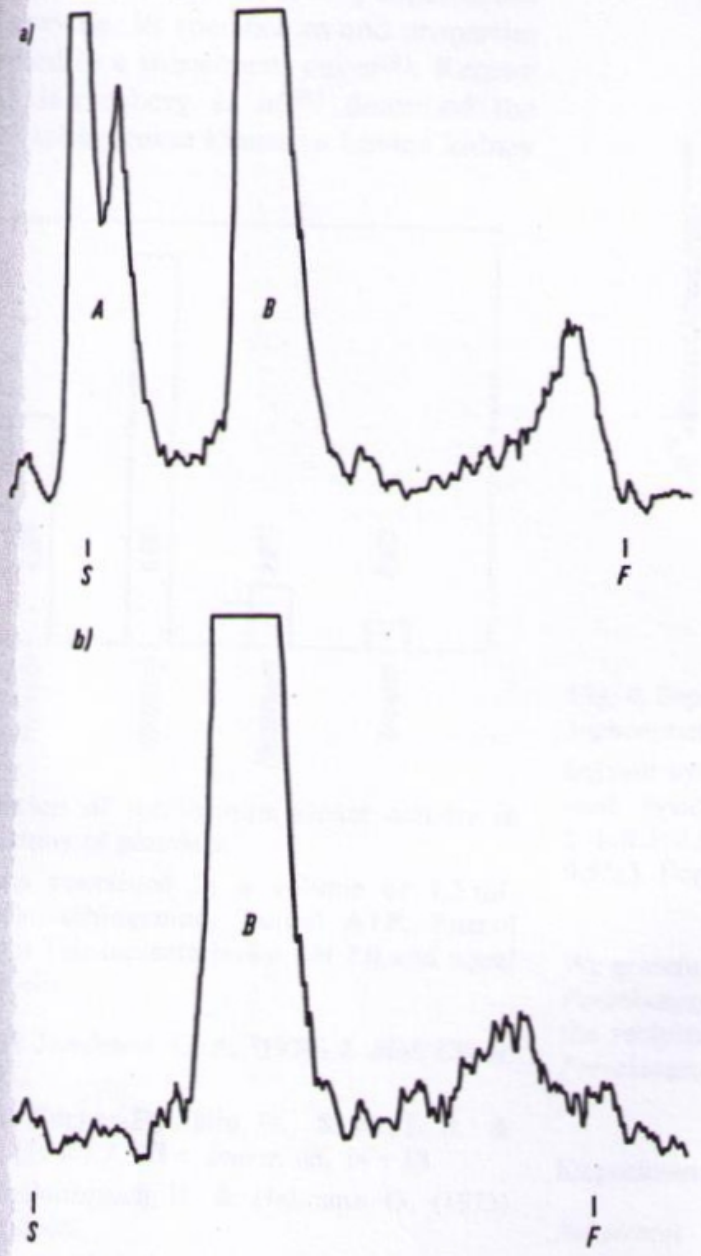


Fig. 1. Radio thin-layer chromatograms of the lipids extracted from the incubation medium after incubating a) platelets and b) erythrocytes with [3-$^{14}$C]sphinganine. Solvent system: chloroform/methanol/water 65:25:4.

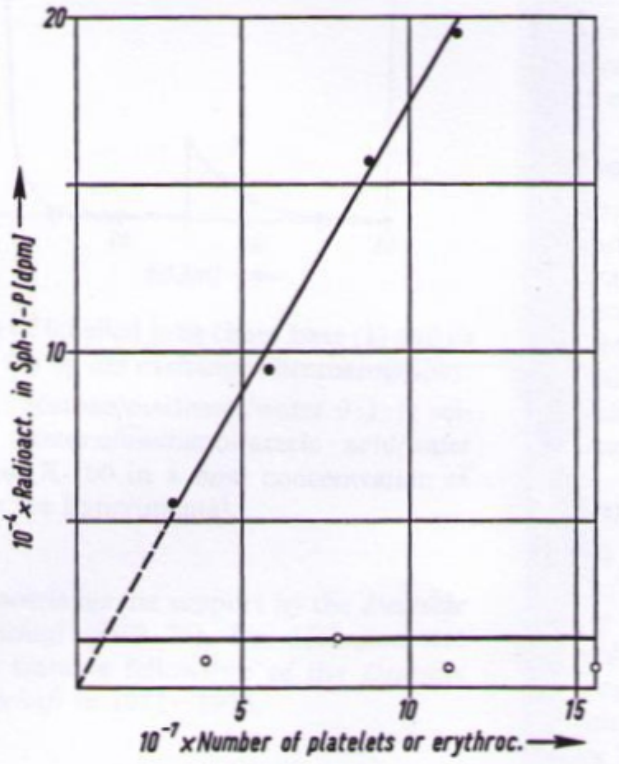


Fig. 2. Correlation of number of platelets and erythrocytes with sphinganine 1-phosphate synthesis. Each incubation contained the number of platelets (●——●) or erythrocytes (○——○) as indicated on the abscissa. Ordinate: radioactivity in sphinganine 1-phosphate (Sph 1-*P*). Conditions were those described under Experimental.

thrombocytes (Fig. 3). Platelets were fractionated into membranes prepared according to Barber *et al.*[2], granules prepared according to Marcus *et al.*[3] and 100000 × *g* supernatant as described under Experimental. Each fraction was incubated with [3-$^3$H]D(+)*erythro*-sphingenine under the same conditions. The qualitative analysis of the incubated materials by thin-layer chromatography proved that the supernatant fraction yielded by far the greatest amount of 4*t*-sphingenine 1-phosphate. A quantitative comparison of the enzyme activity in whole platelets, 100000 × *g* supernatant (0.49 mU), membranes (0.077 mU) and granules (0.03 mU) supported these analyses. The kinase is present in the 100000 × *g* supernatant as a soluble enzyme. Its specificities and properties will be described in a subsequent paper[4]. Keenan *et al.*[5] and Hirschberg *et al.*[6] described the presence of a sphingenine kinase in bovine kidney and rat liver. Both authors also described the kinase as a soluble enzyme.

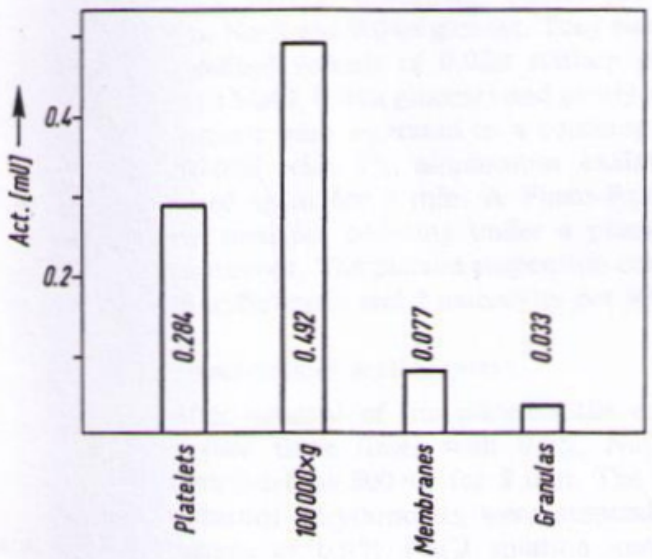


Fig. 3. Distribution of sphinganine kinase activity in subcellular fractions of platelets.

The incubations contained in a volume of 1.5 m*l*: 0.3 μmol [3-$^3$H]4*t*-sphingenine, 7 μmol ATP, 7 μmol $MgCl_2$, 150 μmol Tris-maleate buffer pH 7.0 and equal amounts of protein.

The quantitative analyses during the enzymatic studies were facilitated by the technique applied to the separation of the substrates (the long chain bases) from the product, the rather insoluble 1-phosphate esters of the long chain bases. The two compounds are adsorbed on a Dowex anion exchange resin. The unreacted substrate is then eluted first and separated quantitatively from the phosphate ester which is desorbed and dissolved by the addition of Triton X-100 to the eluting solvent. Fig. 4 depicts a separation.

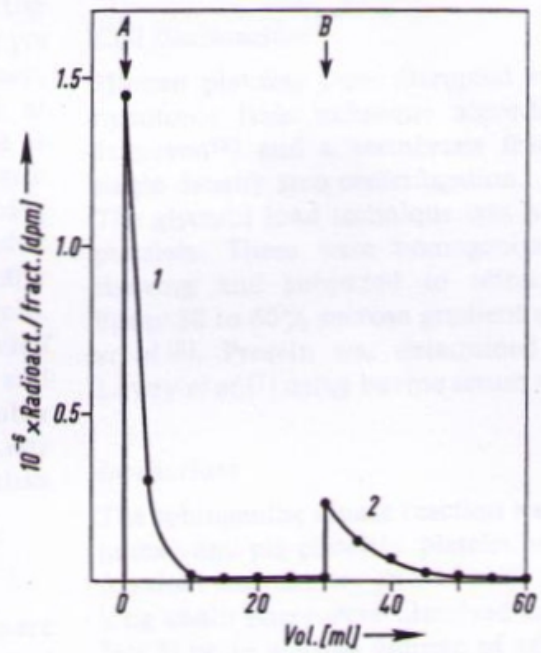


Fig. 4. Separation of labelled long chain base (1) and its 1-phosphate ester (2) by ion exchange chromatography. Solvent system A: acetone/methanol/water 9:1:1; solvent system B: acetone/methanol/acetic acid/water 1:1:0.5:0.5 (Triton X-100 in a final concentration of 0.5%). For details see Experimental.

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## Experimental

### *Substrates*

The following substrates were used in the studies described here: [3-$^3$H]4*t*-sphingenine (specif. radioactiv. $1.2 \times 10^7$ dpm/μmol), [3-$^{14}$C]DL-sphinganine specif. radioactiv. $4.7 \times 10^5$ dpm/μmol) and [3-$^3$H]D-

[2] Barber, A. J. & Jamieson, G. A. (1970) *J. Biol. Chem.* **245**, 6357 – 6365.

[3] Marcus, A. J., Zucker-Franklin, D., Safier, L. B. & Ullmann, H. L. (1966) *J. Clin. Invest.* **45**, 14 – 18.

[4] Stoffel, W., Hellenbroich, B. & Heimann, G. (1973) *this J.* in preparation.

[5] Keenan, R. W. & Haegelin, B. (1969) *Biochem. Biophys. Res. Commun.* **37**, 888 – 894.

[6] Hirschberg, C., Kisic, A. & Schroepfer, G. J., Jr. (1970) *J. Biol. Chem.* **245**, 3084 – 3090.

*erythro*-sphinganine (specif. radioactiv. $3.4 \times 10^6$ dpm/μmol). These compounds were synthesized in this laboratory.

*Preparation of human platelets*

500 m*l* of freshly collected human blood, containing acid citrate-dextrose (USP formula A) as anticoagulant, had been generously supplied by Prof. Bube (Blutbank der Universität Köln). Blood platelet rich plasma was prepared by a centrifugation step at $840 \times g$ for 7 min at ambient temperature. In a second centrifugation the contaminating erythrocytes and leucocytes were sedimented at $680 \times g$ for 10 min using special tubes with a tapered bottom (3 cm long and 3 mm in diameter). The platelets sedimenting on top could be easily removed from the erythrocyte-leucocyte pellet. The supernatant from this centrifugation step, still containing platelets, was finally centrifuged at $2400 \times g$ for 20 min in a Sorvall RC 2-B centrifuge at 4°C. The combined platelets were washed twice with 0.02M sodium phosphate buffer, pH 7.4 containing 0.1M NaCl and 0.04M glucose. They were sedimented in a defined volume of 0.02M sodium phosphate buffer (0.1M NaCl, 0.04M glucose) and gently mixed for 3 min. Portions were aspirated in a counting pipette, diluted 200-fold with 1% ammonium oxalate solution and mixed again for 3 min. A Fuchs-Rosenthal chamber was used for counting under a phase contrast Leitz microscope. The platelet suspension contained less than 10 erythrocytes and 2 leucocytes per $10^5$ platelets.

*Preparation of erythrocytes*

After removal of the platelets the erythrocytes were washed three times with 0.9% NaCl solution and centrifuged at $800 \times g$ for 5 min. The supernatant was discarded. Erythrocytes were suspended in a defined volume of 0.9% NaCl solution and, after dilution (200-fold) they were counted in a Fuchs-Rosenthal chamber. The contamination by platelets was less than 1%.

*Preparation of pig platelet homogenate*

All glassware used was siliconized (Siliconöl WS 60, Fa. Wacker-Chemie, München, W. Germany). 10 *l* of pig blood was collected in a plastic bottle containing acid citrate-dextrose solution (USP formula A) as anticoagulant (67.5 m*l* to 450 m*l* of blood). The platelet rich plasma was prepared by centrifugation at $840 \times g$ for 7 min in a Stock centrifuge at ambient temperature. In a second centrifugation cycle erythrocytes and leucocytes were sedimented at $680 \times g$ for 10 min in a Runne centrifuge in tapered tubes. The platelets were carefully removed from the erythrocyte pellet. The supernatant containing the platelets was finally centrifuged at $2400 \times g$ for 20 min in a Sorvall RC 2-B centrifuge at 4°C. The combined platelets were washed twice with 0.02M sodium phosphate buffer pH 7.4, containing 0.1M NaCl and 0.04M glucose. The final suspension contained less than 10 erythrocytes and 2 leucocytes per $10^5$ platelets. The washed platelets were then frozen at −18°C. They could be stored in this way for several weeks without significant loss of enzymic activity. The platelets were lysed by thawing at 20°C for 30 min under hypotonic conditions (0.02M sodium phosphate buffer pH 7.4). The platelet homogenate was then centrifuged in the SW 27 rotor of a Beckman model L 2 65 B ultracentrifuge at 4°C at 27000 rpm ($131000 \times g$) for 60 min. The supernatant was stored at 4°C. The freezing and thawing were repeated in the same manner. In this way additional enzyme activity was released.
Similar studies were carried out with *human platelets*.

*Cell fractionation*

Human platelets were disrupted by the glycerol load hypotonic lysis technique according to Barber and Jamieson[2] and a membrane fraction isolated by a single density step centrifugation.
The glycerol load technique was not applicable to pig platelets. These were homogenized by freezing and thawing and subjected to ultracentrifugation on a linear 30 to 60% sucrose gradient according to Marcus *et al.*[3]. Protein was determined by the method of Lowry *et al.*[7] using bovine serum albumin as standard.

*Incubations*

The sphinganine kinase reaction was studied with intact human and pig platelets, platelet homogenate, granular fraction, membranes and $100000 \times g$ supernatant. The long chain bases were dissolved either in saline (0.9% NaCl) or in a small volume of ethanol. The standard incubation mixture contained in a total volume of 1.0 m*l*: 0.3 μmol labelled 4*t*-sphingenine or sphinganine, 80 μmol Tris-maleate buffer pH 7.0, 7 μmol $MgCl_2$, 7 μmol ATP, 10 μmol 2-mercaptoethanol and 3−7 mg protein. After incubation for 90 min at 37°C in a shaking water bath the reaction was stopped by the addition of 2 m*l* of acetone/methanol (1:1). The mixture was extracted at 45°C for 5 min, centrifuged and the protein precipitate reextracted twice with 2 m*l* of acetone/methanol 1:1. The combined extracts were used for the subsequent analytical procedures.

*Analytical procedures*

The combined extracts were transferred to a $0.5 \times 4.0$ cm column of Dowex $1 \times 8$ $OH^{\ominus}$ form, 100−200 mesh, 3.5 mequ/g (Fa. Carl Roth, Karlsruhe, W. Germany). The unreacted substrate (sphinganine) was quantitatively eluted with 30 m*l* of an acetone/methanol/water mixture (9:1:1) (A). The product (sphinganine 1-phosphate) was then eluted with 30 m*l* of the following solvent

[7] Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265−275.

system: acetone/methanol/acetic acid/water 1:1:0.5:0.5) and Triton X-100 at a final concentration of 0.5% (B). 5 ml fractions were taken.

Previous separation studies with authentic [3-$^{14}$C]-sphinganine and [3-$^{3}$H]sphinganine 1-phosphate had revealed that these two substrates separated quantitatively and reproducibly under the conditions described above. The error inherent in the method was within ± 1%. The eluate was taken to dryness at 45°C under reduced pressure and the residue dissolved in 2 ml of chloroform/methanol (2:1). Portions were counted in a Packard Tricarb liquid scintillation spectrometer, model 3380. The toluene based scintillation fluid contained 5.0 g 2,5-diphenyloxazol and 0.3 g of 1,4-bis-[4-methyl-5-phenyl-2-oxazolyl]benzene/l.

*Identification and characterization of [1-$^{3}$H]4t-sphingenine 1-phosphate*

a) The radioactive sphingenine 1-phosphate exhibited its well known chromatographic behavior in thin-layer chromatography; i. e. in the solvent system 1: chloroform/methanol/water 65:25:4, $R_F$: 0.0; while in solvent system 2: n-butanol/acetic acid/water 6:2:2, $R_F$: 0.45−0.50. It was detected by radio thin-layer chromatography using either the Packard radiochromatogram scanner, model 7201 or the Berthold scanner, model LB 2722.

b) Periodate oxidation was carried out as follows: 0.2 μmol [3-$^{3}$H]4t-sphingenine 1-phosphate ($5 \times 10^6$ dpm) and 1 mg Triton X-100 were dissolved in 2.5 ml of methanol and 0.5 ml of 0.2M $NaIO_4$ was added. The mixture was maintained at 20°C for 60 min in a shaking water bath. After 45 min 1.5 ml of water was added and the mixture extracted with 6 ml of methylene chloride. The upper phase was reextracted once with 6 ml of methylene chloride. The combined organic phases were concentrated and subjected to thin-layer chromatography (solvent: 1,2-dichloroethane, $R_F$ of the long chain aldehydes: 0.45−0.50).

The radioactive aldehyde was localized by the scanning technique, recovered by the technique of Goldrick and Hirsch[8] and identified by radio gas-chromatography on 15% EGS, column length 200 cm, column temperature 155°C, argon flow rate 30 ml/min. The radioactive bands were localized by discontinous sampling of the effluent carrier gas.

*c) Platelet phosphate reaction*

The platelet phosphatase reaction was studied with pig platelet homogenate, the granular fraction, membrane fraction and 100000 × g supernatant. Each incubation mixture contained in a total volume of 0.5 ml: 0.05 μmol [3-$^{3}$H]4t-sphingenine 1-phosphate ($1.25 \times 10^6$ dpm), 0.25 mg Triton X-100, 25 μmol Tris-maleate buffer pH 7.1 and 2 to 4 mg of protein. After 60 min at 37°C the incubation was stopped by addition of 2 ml of a chloroform/methanol mixture (2:1). After centrifugation the aqueous phase was reextracted with chloroform/methanol (2:1). The lipid extracts were analyzed by radio thin-layer chromatography as described before (solvent system: chloroform/methanol/water 65:25:4).

[8] Goldrick, B. & Hirsch, J. (1963) *J. Lipid Res.* **4**, 482−483.