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The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity

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Abstract

Allicin (diallyl thiosulfinate) is the main biologically active component of the freshly crushed garlic extracts. In the present work the ability of allicin to cross through membranes (artificial and biological) was studied. Partition coefficients of allicin in water/octanol, water/hexadecane and water/phospholipids mixtures were determined. Using phospholipid vesicles loaded with hydrophilic thiols (reduced glutathione or 2-nitro-5-thiobenzoate), we observed that allicin freely permeates through phospholipid bilayers and interacts with the SH groups. The reaction rate of allicin with SH containing molecules after crossing the membrane was the same as in solution. Fast diffusion and permeation of allicin across human red blood cell membranes was also demonstrated. Allicin does not induce leakage, fusion or aggregation of membrane. The high permeability of allicin through membranes may greatly enhance the intracellular interaction with thiols.

Keywords: Allicin; Biological membrane; Penetration

1. Introduction

Many beneficial health related biological effects of garlic (*Allium sativum*) are attributed to its characteristic organosulfur compounds [1–4]. The best known and most extensively studied is allicin (diallyl thiosulfinate), the principal active substance of fresh garlic extract, which is responsible for garlic's typical pungent smell. Allicin is produced according to Scheme 1, during the crushing of garlic cloves by the interaction between the non-protein amino acid alliin and the enzyme alliinase [5].

Allicin is a precursor of a number of secondary products formed in aged garlic and crushed garlic preparations. Allicin possesses various biological activities among which antibacterial, antifungal and antiparasitic effects are included [1–12]. In addition to that it reduces serum cholesterol and triglyceride levels as well as atherosclerotic plaque formation and platelet aggregation, it inhibits cancer promotion and decreases ocular pressure [1–3,13]. Allicin rapidly disappears after injection into the blood [14,15]. This rather unstable compound has been suggested by Lawson anc coworkers to transform rapidly into sec-

Abbreviations: Biradical, bis(2,2,5,5-tetramethyl-3-imidazoline-1- oxyl-4-yl) disulfide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); ESR, electron spin resonance; GSH, reduced glutathione; GSSA, *S*-allylmercaptoglutathione; NTB, 2-nitro-5-thiobenzoate; QELS, quasi-elastic light scattering; RBC, red blood cell; SUV, small unilamellar phospholipid vesicle; TCA, trichloroacetic acid

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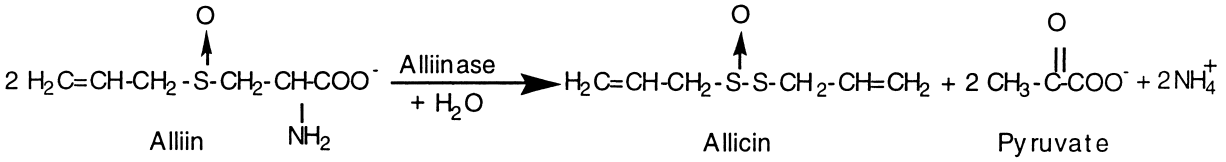


Scheme 1.

ondary products (in vivo), such as allylmercaptan and others [2,14]. At present a variety of biological effects of allicin is attributed to both antioxidant activity and modification of SH-dependent activities [16,17]. These activities have been confirmed by us in model systems [18,19]. Recently the inhibitory effect of allicin on NO formation was also demonstrated [20]. In addition to that, allicin affects the processing of DNA and RNA synthesis [11], signal transduction and apoptosis. All the effects described above are mainly intracellular. Therefore the question arose: how does allicin permeate through the plasma membrane and take part in intracellular processes? In the present work we studied the permeability of allicin through lipid bilayers of phospholipid vesicles as well as through natural membranes of red blood cells (RBCs), using its interaction with internally entrapped thiol containing compounds such as glutathione (GSH) or 2-nitro-5-thiobenzoate (NTB).

2. Materials and methods

L-Cysteine, reduced glutathione, oxidized glutathione, dimyristoylphosphatidylcholine (DMPC), calcein and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Egg phosphatidylcholine (PC) was purchased from Lipid Products (South Nutfield, UK). Cholesterol (extra pure) was from Merck (Darmstadt, Germany). Allicin was prepared enzymatically from synthetic alliin and purified by HPLC [18]. NTB was prepared according to [21]. Symmetrical stable nitroxyl biradical containing disulfide bond, bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl) disulfide (Biradical), synthesized according to [22], was a kind gift of Dr. V. Martin (Lipitek Int. Inc., San Antonio, TX, USA). All other reagents were of analytical grade.

2.1. Synthesis and isolation of a S-allylmercaptoglutathione (GSSA)

The reaction between allicin and GSH was performed using an excess of allicin. GSH (200 mg in 5 ml water, pH 6.0) was added dropwise to allicin (130 mg), dissolved in 50% methanol (2 ml) and kept at room temperature for 2 h. The product of the reaction was detected by HPLC analysis. Excess allicin was removed by extraction with ether. The water phase was dried by lyophilization. The product was re-dissolved in water and dried again by lyophilization. The structure was confirmed by NMR and MS.

2.2. Assay of free sulfhydryl groups

Determination of free sulfhydryl groups was done with DTNB by using ε_M 14 150 M^{-1} cm^{-1} at 412 nm according to [23]. Determination of free SH in human RBCs was also done with Biradical using electron spin resonance (ESR) as described by Weiner [24].

2.3. Determination of partition coefficient of allicin

Determination of partition coefficients of allicin in phospholipids, octanol and hexadecane was done according to [25]. Briefly, allicin (6.5 mM, 250 μl) in 0.05 M Na phosphate buffer (pH 6.5) was added to dry lipid (10 mg) or solvent (10 μl). The allicin/lipid dispersions were homogenized by vortexing and shaking for 1 h at 37°C (in the case of phospholipid) or at room temperature (in the case of the solvents). The amount of remaining allicin in the water phase was determined by HPLC. The partition coefficient (K_p) of allicin (solvent/water or lipid/water) was defined as follows:

$$K_p = \frac{[\text{allicin}]_{\text{lipid}}}{V_{\text{lipid}}} \times \frac{V_{\text{water}}}{[\text{allicin}]_{\text{water}}} \quad (1)$$

where $[\text{allicin}]_{\text{lipid}} = [\text{allicin}]_{\text{total}} - [\text{allicin}]_{\text{water}}$, V_{water} and V_{lipid} are the volumes of the water phase and the lipid or solvent phase respectively.

2.4. Separation of alliin, allicin and GSSA by HPLC

Quantitative determinations of alliin, GSSA and allicin were performed in an LKB HPLC system with the SP 4290 integrator (Spectraphysics). The separation was achieved on a LiChrosorb RP-18 (7 μm) column using 60% methanol in water containing 0.1% formic acid as an eluant. Flow rate was 0.56 ml/min.

2.5. Determination of rate constants

The rate constant for the reaction of allicin and GSH was obtained in 0.1 M NaCl, 0.01 M Na phosphate buffer (pH 7.0). The initial rate of GSSA appearance was monitored by HPLC. At various time intervals, samples were diluted with HPLC running buffer and analyzed by HPLC. For calculation the following equation was used:

$$d[\text{GSSA}]/dt = K[\text{Allicin}] \times [\text{GSH}] \quad (2)$$

where $d[\text{GSSA}]/dt$ is the initial rate of GSSA appearance, K is the bimolecular rate constant, [Allicin] and [GSH] are the initial concentrations of allicin and GSH.

The rate constant for the reaction of allicin and NTB at pH 7.0 was obtained from the product formation dependence on temperature. The concentration ranges used were: NTB $(1–7)\times10^{-5}$ M, allicin $(2–30)\times10^{-6}$ M. The reaction was carried out in a thermostated reaction cell in a HP8453 spectrophotometer under constant mixing.

2.6. Preparation of phospholipid vesicles

Small unilamellar phospholipid vesicles (SUV) were made either from egg phosphatidylcholine (PC) or from dimyristoylphosphatidylcholine (DMPC) and cholesterol. In the case of SUV made from PC, the concentration of cholesterol was 17% mol/mol. In the case of SUV made from DMPC, cholesterol content was 25% mol/mol. Cholesterol was dissolved in $CHCl_3$/MeOH (2:1 v/v). The cholesterol solution was added to the dry lipid. A film of 2.5–5 mg lipid/glass vial was prepared by evaporating the solvents under a stream of nitrogen, followed by 3 h drying under high vacuum. SUV containing either GSH, calcein, calcein-cobalt complex, EDTA, or NTB were obtained by sonication according to [26]. SUV containing GSH were prepared as follows: GSH solution (0.2 M in 0.05 M NaCl, pH 7.0) was added to the dried film, mixed by vortex and sonicated for 10–30 min in a bath-type sonicator (G1125SP1, Laboratory Supplies Co. Inc., New York, NY, USA). Final phospholipid concentration was either 5 or 10 mg/ml. Other vesicles were prepared in a similar way. Solutions used were of the following concentrations: 0.1 M Na_2CO_3 (pH 7.0) containing calcein 60 mM or 60 mM calcein plus 160 mM $CoSO_4$; 0.03 M NaCl containing EDTA 0.25 M (pH 8); 0.03 M NaCl in 0.01 M Na phosphate buffer (pH 7.0) containing 50 mM NTB.

2.7. Gel filtration

Separation of SUV from free reagents was done by gel filtration on Sephadex G-50 (15 ml bed volume) pre-equilibrated with 0.1 M NaCl in 0.01 M Na phosphate buffer (pH 7.0). In the experiments with calcein-cobalt complex and EDTA, the gel was equilibrated with 0.2 M NaCl in 10 mM Na phosphate buffer (pH 7.0). Vesicles were eluted in the void volume and were monitored by absorption at 340 nm.

2.8. Measurements of leakage, fusion and aggregation of liposomes

Allicin was tested for its potency to cause leakage from SUV. These experiments were performed with vesicle entrapped calcein, the fluorescent probe [27]. The intrinsic fluorescence of entrapped and water diluted calcein was monitored by Shimadzu RF-540 spectrofluorometer. Excitation wavelength was 490 nm, emission range 500–600 nm. A fluorescence assay was performed to check the possible fusogenic effect of allicin on SUV [28]. For this purpose SUV (DMPC/cholesterol) loaded with EDTA and gel filtered through Sephadex G-50 column (equilibrated with 0.2 M NaCl, 0.01 M Na phosphate buffer (pH 7.0) were mixed with SUV (DMPC/cholesterol), preloaded with a complex of cobalt-calcein and gel filtered through Sephadex G-50 column equilibrated

with 0.2 M NaCl, 0.01 M Na phosphate buffer (pH 7.0). The fluorescence was monitored for a few minutes before adding allicin (baseline), and until 4 min after adding it to the vesicle mixture (10^{-4} M final concentration). At the end of the experiment Triton X-100 (0.2% final concentration) was added to destroy the vesicles and to get the full value of fluorescence. There was no constant mixing of the sample in the spectrofluorometer cell.

2.9. Preparation of red blood cells

Fresh human blood (in the presence of heparin) was washed three times with phosphate buffered saline (PBS). RBCs were collected after centrifugation (2000 rpm, 5 min) and resuspended in PBS. A 10% or 1% (v/v) suspension of RBC in PBS was used in the experiments.

2.10. ESR experiments

Measurements were performed in a flat cell of the Bruker ER-200 D-SRC spectrometer. The experimental conditions included the following: field, 3500 G; sweep width, 100 G; receiver gain, 2×10^5; microwave power, 20 mW; modulation amplitude, 0.8 G.

2.11. NMR spectroscopy

NMR spectra were collected on a Bruker AMX-400 spectrometer. The product of the interaction between allicin and glutathione was dissolved in deuterated water. ^{1}H and ^{13}C spectra were collected at 25°C. Structure analysis of the product obtained was performed as described previously [18,19]

Table 1
Partition coefficients (K_p) for allicin in phospholipid/H_2O and organic solvents/H_2O

Lipid or solvent	Temperature (°C)	K_p
DMPC	37	4.36 ± 0.50
Hexadecane	24	1.77 ± 0.31
Octanol	24	12.35 ± 1.06
PC	37	10.97 ± 0.39

The results represent the mean value of three independent experiments.

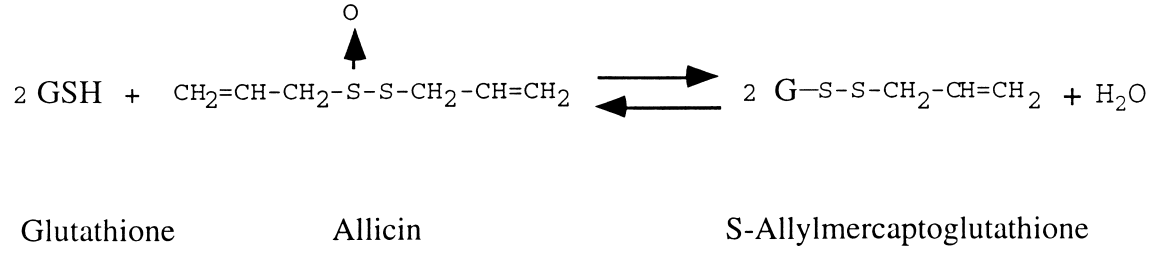
2.12. Light scattering measurements

The size of SUV was measured by the quasi-elastic light scattering (QELS) technique as described in [29]. Thin-wall cylindrical cells (outer diameter, 10 mm) were employed and measurements were performed at 0.5 mg of lipid/ml at 22°C. Allicin and the buffer solution were filtered prior to measurements using 0.22 μm pore diameter Durapore filters (Millipore Corporation, Bedford, MA).

3. Results

3.1. Determination of partition coefficients (K_p) for allicin in lipid/H_2O and organic solvent/H_2O

Knowledge of the partition coefficient in a lipid/water mixture is a valuable parameter for evaluation of possible interactions of various compounds with lipid membranes. We measured the K_p of allicin in octanol/H_2O, hexadecane/H_2O and two phospholipids (PC and DMPC)/H_2O mixtures. K_p values, calculated according to Eq. 1 are presented in Table 1. In the case of PC or DMPC, the measurements were



Scheme 2.

performed at temperatures maintaining the phospholipids in the liquid crystalline state. According to these data, allicin is a fairly hydrophobic agent, easily dissolved in octanol as well as in the two phospholipids used in this work, and less soluble in hexadecane.

3.2. Reaction of allicin with GSH

SUV containing GSH were prepared in order to show allicin's propensity to permeate the lipid bilayer membrane. GSH, the main intracellular thiol of mammalian cells, is highly hydrophilic and therefore cannot pass lipid membranes [30,31]. GSH, like other thiols [6,18,19], reacts rapidly with allicin as presented in Scheme 2.

The reaction between GSH and allicin was followed by HPLC and the product was characterized by ^{1}H and ^{13}C NMR as described earlier [18,19]. The elution peak of the reaction product, GSSA (retention time 5.3 min), is positioned between that of allicin (retention time 8 min) and GSH (retention time 4.6 min). The kinetics of the reaction between GSH and allicin ($\sim$2:1 ratio) in water is shown in Fig. 1. Formation of the product and disappearance of allicin were determined at various time intervals. The reaction was also monitored at different concentrations of allicin and GSH and at different temperatures, bimolecular rate constant values, calculated according to Eq. 2, are presented in Table 2.

3.3. Interaction of allicin with GSH entrapped in SUV

Allicin reacts with GSH entrapped in liposomes at a high rate. The rate of GSSA formation in the vesicles (Table 2) was only slightly slower than in solution. The vesicle composition, DMPC or PC, shows no significant effect on allicin permeability. A decrease in temperature to less than 23°C, below the phase transition of DMPC/cholesterol vesicles, did not hinder allicin penetration. These findings indicate that allicin permeates very easily through the lipid bilayers.

3.4. The reaction of allicin with NTB

Monitoring of the allicin interaction with GSH by HPLC has the disadvantage of inaccurate initial rate measurements of the reaction during the first 5–15 s (at pH 7.0). Therefore we used NTB as a hydrophilic colorimetric reagent which not only reacts with allicin (according to Scheme 2 [19]) but also stays within the vesicle, not being able to penetrate through the phospholipid bilayer [32]. The kinetics of the reaction can be measured immediately after mixing of allicin with NTB. The kinetics of the decrease in NTB absorbance was monitored at different concentrations of allicin and NTB, at two temperatures, 12°C and 30°C, in solution and in vesicles containing NTB (see Fig. 2). The bimolecular rate constants of the reaction of NTB with allicin are presented in Table 3.

3.5. Effect of allicin on leakage, fusion and aggregation of vesicles

The reaction of allicin with SH reagents located in the internal volume of the vesicles raises the question whether allicin affects the membrane integrity, and

Table 2
Bimolecular rate constants for the reaction of allicin with GSH entrapped in phospholipid vesicles or in solution

	[GSH] 10^{-3} M	Allicin 10^{-3} M	Temperature (°C)	K (M^{-1} s^{-1})
Solution	0.22	0.80	4	26.28 ± 1.25 (3)
	0.22	0.80	24	48.66 ± 2.45 (3)
	0.42	0.58	35	88.13 ± 5.32 (3)
Vesicles: DMPC/cholesterol 3:1 mol/mol	0.18	2.90	10	21.29 ± 2.20 (2)
	0.34	1.35	15	23.38 ± 2.75 (2)
	0.34	1.35	24	33.57 ± 4.95 (2)
	0.34	1.35	35	78.45 ± 4.13 (2)
PC:cholesterol 5:1 mol/mol	0.16	1.92	24	30.40 ± 2.47 (2)
	0.11	0.68	24	31.28 ± 3.15 (2)

Reaction was performed in 0.1 M NaCl, 0.01 M Na phosphate buffer (pH 7.0). The results are the mean values of n independent experiments, as indicated in parentheses.

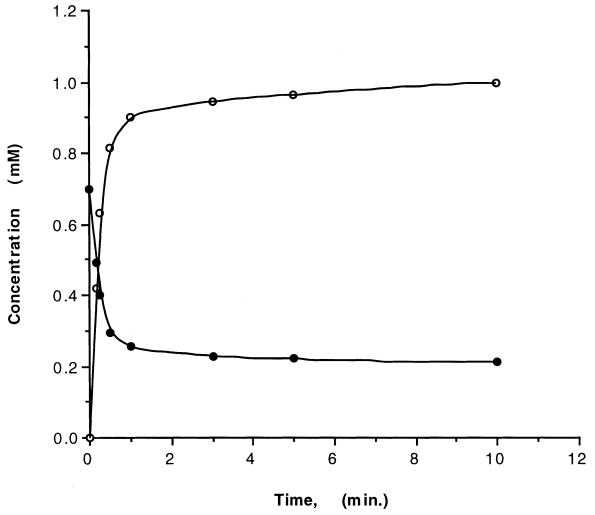


Fig. 1. Kinetics of the reaction of allicin with GSH, monitored by appearance of *S*-allylmercaptoglutathione (○) and disappearance of allicin (●). The initial concentration of GSH was 1.2 mM and that of allicin 0.7 mM. The reaction was carried out at pH 7.0.

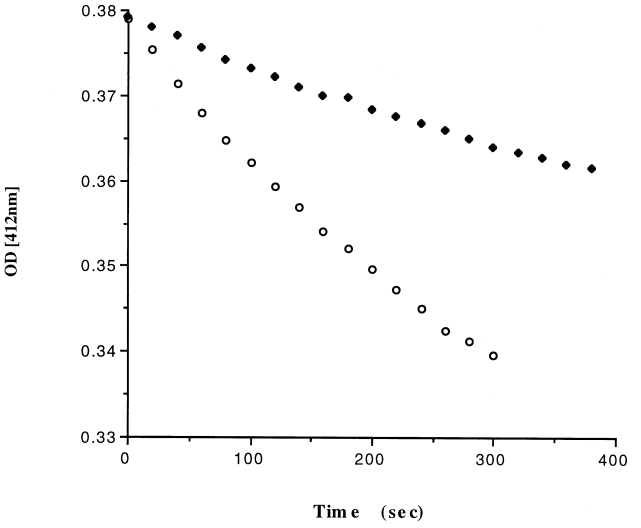


Fig. 2. Kinetics of the reaction of allicin and NTB in liposomes (DMPC/cholesterol 3:1 mol/mol) as a function of temperature. Allicin/NTB at 12°C (♦), allicin/NTB at 30°C (○). The initial NTB concentration in the liposomes was 27 μM, that of allicin was 7.2 μM.

whether it perturbs the structure of the bilayers. Membrane active compounds most commonly induce membrane fusion and leakage. To test the possibility of fusion and/or leakage of vesicles, we examined the effect of allicin on the permeability of DMPC/cholesterol vesicles using calcein loaded in vesicles. If calcein is entrapped in lipid vesicles at a high concentration, it displays a very low fluorescence intensity due to self quenching. As the probe is released from the vesicles, the local concentration of calcein decreases and the amplitude of the fluorescence signal increases, due to the dilution of calcein in the bulk surrounding phase. This technique enabled us to show that there was no leakage from DMPC/cholesterol vesicles in the absence and presence of 1×10^{-3} M allicin (data not shown). For testing allicin effects on fusion of vesicles, we used vesicles loaded either with Co(II)-calcein or with EDTA [28]. No significant fluorescence was observed when allicin was injected into the solution containing the mixture of these vesicles (Fig. 3). However, addition of Triton X-100 (final concentration 0.2%) to

Table 3
Bimolecular rate constants of the reaction of allicin with NTB in solution and entrapped in vesicles

Temperature (°C)	[Solution] (M^{-1} s^{-1})	[SUV: DMPC/cholesterol (3:1)] (M^{-1} s^{-1})
12	49.63 ± 2.89	27.06 ± 1.14
30	119.76 ± 5.69	79.44 ± 5.39

Reaction was performed in 0.1 M NaCl, 0.01 M Na phosphate buffer (pH 7.0). The results are the mean value of three independent experiments.

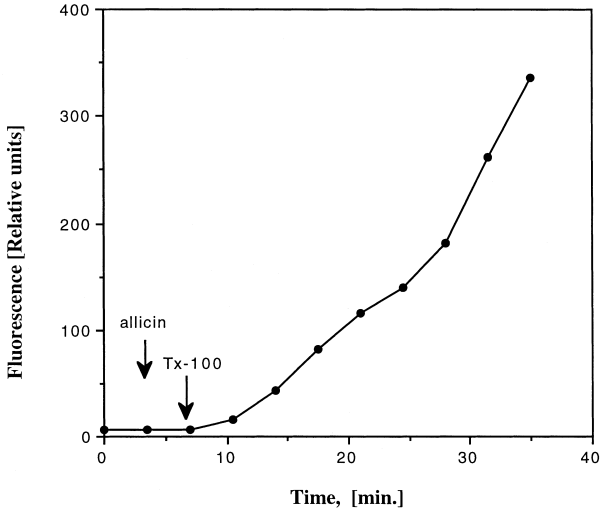


Fig. 3. Addition of allicin to a mixture of liposomes (DMPC/cholesterol 3:1 mol/mol) containing either EDTA or calcein-cobalt. Arrows show the time of injection of allicin and Triton X-100 respectively.

Table 4
Recording of the reaction product GSH+allicin, after gel filtration of vesicles containing GSH treated with allicin

Vesicles	[GSSA] in vesicles 10^{-3}M	Yield (%)
A. DMPC/cholesterol (3:1 mol/mol, 10 mg phospholipid/ml)		
Before second gel filtration	0.404	100
After second gel filtration	0.375	92.8
B. PC:cholesterol (5:1 mol/mol, 5 mg phospholipid/ml)		
Before second gel filtration	0.278	100
After second gel filtration	0.234	79.9

the mixture caused a drastic increase of fluorescence (see Fig. 3). These results show that allicin by itself has no effect on leakage and fusion of vesicles. The possibility of leakage from internal volume of vesicles containing either GSH or NTB after treatment of the SUV with allicin was also examined. The control experiment was done as follows: vesicles containing GSH were reacted with allicin (1×10^{-3} M) for 1 min at 15°C and GSSA was determined. A freshly prepared cysteine solution (1×10^{-2} M) was added to block external allicin and the mixture was gel filtered at room temperature on Sephadex G-50 as described in Section 2. GSSA was determined again in the SUV fractions. The results, shown in Table 4A (DMPC/cholesterol vesicles), indicate that no significant leakage nor pore formation was observed in the vesicles after allicin treatment. The same experiment was then performed with PC/cholesterol vesicles containing GSH at pH 7.0. No significant leakage was observed in this case either (Table 4B).

QELS was used to investigate if allicin causes aggregation of vesicles. QELS measurements show that allicin has no effect on the size of vesicles at the concentration of 1.5×10^{-3} M. This finding shows that allicin does not cause aggregation of vesicles at the concentration used in this experiment.

Table 5
Concentration of intracellular thiols (R-SH) determined in human RBC

Method	[R-SH] in human RBC
Biradical (ESR)	1.78×10^{-3} M ± 0.20
DTNB[a]	1.69×10^{-3} M ± 0.34

The results are means of three independent experiments.
[a]Assay of R-SH was done after protein TCA precipitation of RBC suspension, and neutralization of the supernatant to pH 7.0 with NaOH.

3.6. Interaction of allicin with red blood cells

Red blood cells are naturally occurring membrane vesicles in which GSH is present at a concentration of 1–2 mM [24,33]. The amount of thiols in PBS washed human RBC was measured either by the noninvasive ESR technique with Biradical developed earlier by Weiner [24], or directly after TCA precipitation of the RBC and assay with DTNB at pH 7.0 (Table 5). The total concentrations of thiol containing compounds estimated by the two independent methods were very similar (about 2 mM) and are in a good agreement with data reported by others

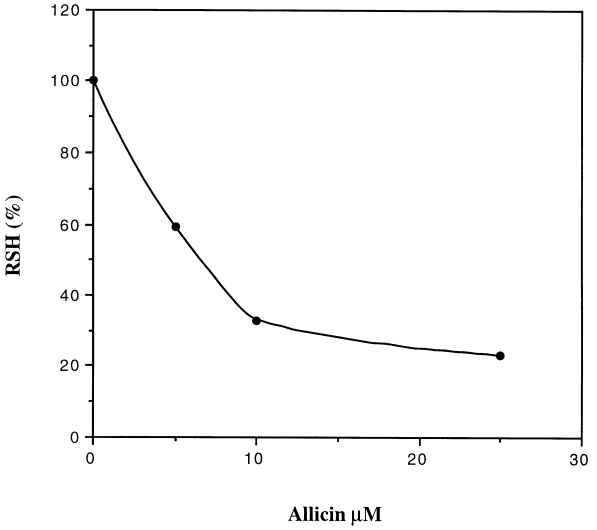


Fig. 4. Estimation of concentration of intracellular thiols under incubation, as a function of allicin added. Concentration of SH groups was estimated by ESR with Biradical in RBC suspension (1%) (see Section 2).

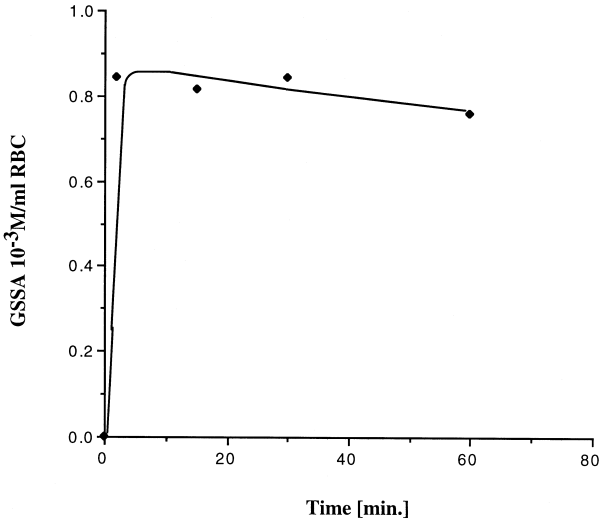


Fig. 5. Kinetics of appearance of GSSA in RBC at 37°C after treatment with allicin. Allicin treated RBC were washed with cysteine and PBS to remove external allicin, then treated with TCA. The concentration of GSSA was assayed in the supernatant.

[33,34]. The advantage of the ESR method is that it enables us to monitor the decrease of thiol concentration in cells after short exposure to allicin. Allicin (up to 2.5×10^{-5} M final concentration) was added to a 1% washed RBC suspension in an Eppendorf tube. After 1–2 min at room temperature, the Biradical was added (10^{-4} M final concentration) and the content of residual thiol concentration was monitored. Allicin penetrates through the RBC membrane and reacts with intracellular thiols in a concentration dependent manner as shown in Fig. 4.

3.7. Identification of reaction products

Washed human RBC were treated at 37°C with allicin (5×10^{-4} M). At different time intervals, a fresh solution of cysteine (20 mM final concentration) was added to block external allicin, and the suspension was washed three times with PBS. The washed RBC were treated with TCA (final concentration 5%), mixed by vortexing and centrifuged. The supernatant was diluted 1:12.5 with HPLC running buffer and analyzed by HPLC. The main product appeared as GSSA, which was formed immediately after allicin penetration into the RBC (Fig. 5). In this experiment, allicin was not in excess with respect to internal thiols.

3.8. Effect of allicin on RBC leakage

Allicin (1×10^{-4} M) was added to RBC (10%) at room temperature, and at various time intervals (up to 1 h) samples were removed and spun down. The spectra of the supernatants were determined. Control experiments without allicin were run under the same condition. No leakage of hemoglobin to the supernatant was observed. Moreover, the color of the red blood cell pellet changed to brown during the incubation with allicin, which might indicate that allicin penetrates the membrane [15].

The fact that there is no leakage of hemoglobin does not exclude the possible release of GSH during treatment of RBC with allicin. A control experiment was carried out in which RBC were treated with allicin (0.5×10^{-4} M) in the presence of DTNB (1×10^{-4} M) for 15 min at room temperature. The RBC were sedimented and the absorbance at 412 nm of the supernatant was determined. No absorbance of NTB (the reaction product of GSH or cysteine with DTNB) and no GSSA were observed in the supernatant.

4. Discussion

We have recently shown that the beneficial effects of allicin on health may stem from its interaction with SH containing molecules such as enzymes and/or its high antioxidant activity [10,18,19]. Free thiol containing compounds are usually intracellular, since extracellular thiols are in most cases oxidized to disulfide by the environmental molecular oxygen. Therefore we investigated the manner in which allicin can penetrate cell membranes and interact with intracellular thiols. Low molecular weight thiol containing compounds such as GSH and NTB were entrapped in lipid vesicles and red blood cells were used as natural containers of GSH. Thus the interaction of the above compounds with externally added allicin could be studied. Based on the kinetics of this reaction, it was clearly shown that the interaction of allicin with the low molecular weight thiols entrapped in the vesicles was not dependent on the release of GSH and NTB from vesicles, since the reaction product, GSSA, was detected in the vesicle

fraction after gel filtration at the end of the reaction (see Table 4).

Comparison of the rates of reaction of allicin with GSH and NTB in solutions with the rates of reaction of these thiols incorporated into internal volume of vesicles (Tables 2 and 3) showed that the lipid membrane decreased the rate of reaction not more than 1.8 times, indicating that allicin easily diffused into the internal volume of the vesicles. Furthermore it was also shown that the kinetics of allicin penetration into artificial vesicles at different temperatures is independent of the phase state of the lipids and has no effect on its interaction rate with thiols[1]. Below and above the phase transition of DMPC vesicles, the rate of the chemical reaction has the same temperature dependence as in solution (see Tables 2 and 3).

This behavior of allicin is very different from the permeability of the small charged molecule superoxide ($O_2^{\bullet -}$ radical) or the hydrophilic glucose molecule into vesicles formed from synthetic lipids. Significant differences in the rate of penetration were observed for these molecules at temperatures below and above the phase transition temperature [38,39]

The mechanism of penetration of small molecules through hydrophobic membrane barriers is still under debate [40,41]. It is assumed that for nonpolar molecules, the major permeation resistance is the barrier rigidity near the membrane surface [42]. We detected no resistance to allicin permeation, which proves barrier rigidity to have no effect, even in the presence of 50% cholesterol.

Permeability coefficients of small hydrophobic molecules in membranes correlate quite well with oil/water partition coefficients. The so-called Overton rule [43], known for almost 100 years, has been verified in numerous experimental systems [41]. For estimation of the permeability coefficient of allicin through the lipid bilayer we studied its partition in an organic solvent/water system [41]. Using the observed value for K_p (Table 1) in a hexadecane/water system, we calculated the allicin permeability coefficient: $P \sim 0.2$ cm s^{-1}. This calculation was based on the known dependence of permeability coefficients on hexadecane/water partition coefficients (shown for a number of low molecular weight compounds and their permeability through RBC membranes) [41]. This value is comparable with the permeation coefficient for *n*-alkanes [41] but is higher than the permeability coefficient for water (2.3×10^{-3} cm s^{-1}) and peroxynitrite ($ONOO^-$) (8.0×10^{-3} cm s^{-1}) [44], or iodoantipyrine ($\sim10^{-3}$ cm s^{-1}) [45].

For the estimation of the diffusion coefficient of allicin across the lipid bilayer we used the following equation [41]

$$P = \frac{K_p D_{mem}}{\lambda} \quad (3)$$

where P is the permeability coefficient, K_p the partition coefficient, λ the thickness of the hydrocarbon region of membrane and D_{mem} the diffusion coefficient within the membrane. (This equation holds for the simplest model, in which the membrane interior is homogeneous and is the rate limiting barrier for transmembrane movement.) Using $\lambda = 50$ Å we obtained a value of D_{mem} for allicin of $\sim5\times10^{-8}$ cm^2 s^{-1}. Interestingly, this value of D_{mem} is very close to the known values for diffusion of nonpolar low molecular weight compounds with molecular weights 2–3 times lower than allicin [41].

In conclusion, the data presented here clearly show that allicin can easily diffuse into the internal volume of vesicles or into the cytoplasm of red blood cells. Lipid bilayers do not constitute a barrier for allicin penetration and its diffusion through the lipid bilayer does not cause membrane leakage, fusion or aggregation. In the case of RBC, there was no creation of pores by allicin in the plasma membrane. These findings raise the possibility that in biological systems allicin can penetrate very rapidly into different compartments of the cells and exert its biological effects. Thus, the significance of allicin as a biological effector molecule is due not only to its high reactivity with low and high molecular weight thiols and its prominent antioxidant activity [10,18,19], but also to its accessibility resulting from high membrane permeability.

[1] Introduction of cholesterol to artificial lipid vesicles broadens the gel-to-liquid crystalline phase transition as well as decreasing the transition enthalpy [35]. However, in the case of DMPC liposomes containing 25 mol% of cholesterol, as was used in this work, the characteristics of phase transition were observed [36,37].

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