INFLUENCE OF BROMOACETYLSULFANILIC ACID AND BROMOACETYL-AMINOISOPHTHALIC ACID ON SULFHYDRYL GROUPS OF EHRLICH ASCITES TUMOR CELLS

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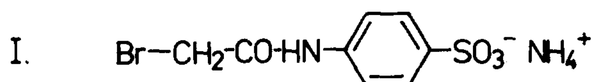
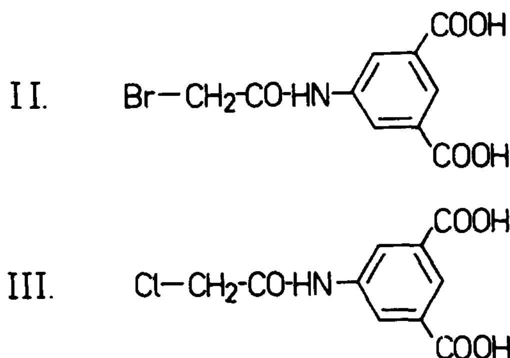
1. Introduction

Previously we reported our experiments on the effect of thiol combining agents such as alkylhalides, maleimides, mercurials and disulfides on the *p*-nitrophenylphosphatase activity of the plasma membrane of Ehrlich Ascites Tumor (EAT) cells [1]. Extending this investigation we sought new SH blocking reagents suitable for studies with intact living cells. Such reagents had to fulfill certain specific requirements. They should react quickly with thiols, because the viability of cells is limited in buffer solutions. The product of the blocking reaction should contain a stable S–C bond; products involving S–S or S-metal bonds are frequently unfavorable because of the possible reversibility of the blocking reaction because of cellular repair mechanisms [2–4]. Finally, reagents were sought having one or more negative charge because this was expected to reduce the penetration of the blocking reagents through the anionic plasma membrane [5] and thus permit a relatively selective reaction with the sulfhydryl components of the cell surface. Three reagents, bromoacetylsulfanilic acid (BASA I) and bromoacetylaminoisophthalic acid (BAAPA II), which meet the above requirements, and chloroacetylaminoisophthalic acid (CAAPA III) were synthesized in this laboratory.





Many different alkyl and aryl halides have been used in biological chemistry as preferred SH reagents [6], but to the best of our knowledge BASA and BAAPA have never been tested with cellular SH functions. Therefore we report the sulfhydryl modification in terms of the effect on the cellular non-protein (acid-soluble) SH level and inhibitory effects on two enzymes, glyceraldehydephosphate dehydrogenase (primarily localized in the cytosol) and *p*-nitrophenylphosphatase (associated with the plasma membrane [1,7]). The reaction of BASA, BAAPA and CAAPA with low molecular SH compounds was also investigated.

2. Materials and methods

2.1. *Substances*

Iodoacetate (IAc), *p*-nitrophenylphosphate

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(*p*NPP), cysteine (Cys), EDTA and buffer substances were from Merck, Darmstadt. Bromoacetyl bromide, chloroacetyl chloride and 5-amino-isophthalic acid were purchased from Merck-Schuchardt, Darmstadt. Iodoacetamide (IAA) was from Sigma, St. Louis USA. Glutathione (GSH), 3-phosphoglycerate, ATP and phosphoglyceratekinase were from Boehringer, Mannheim.

2.1.1. *N*-bromoacetylsulfanilic acid ammonium salt (I): 10.1 g (50 mM) bromoacetyl bromide in 50 ml methylene chloride was added dropwise with stirring to a solution of 5.2 g (30 mM) of sulfanilic acid in 100 ml water while maintaining the pH at 7.5 with a pH-stat. Methylene chloride was removed and the reaction product was isolated by adding 10 g $BaCl_2$ in a few ml of water. The barium salt was separated by filtration and dried; yield 4 g (36%). The ammonium salt was prepared by ion-exchange on a Dowex-50 W × 8 resin (20–50 mesh, ammonium form) and freeze-drying of the eluate.

$C_8H_{11}BrN_2O_4S$ (311.2)

	C	H	N	S
calc.	30.88	3.56	9.00	10.30
found	30.99	3.55	9.01	10.28

2.1.2. *N*-Chloroacetyl- and *N*-bromoacetyl-5-amino-isophthalic acid (III,II):

5-Aminoisophthalic acid was reacted with chloroacetyl chloride or bromoacetyl bromide. Reaction type and scale was the same as described for [1]. After separation of the organic layer and on additional extraction with methylene chloride the reaction products crystallized upon acidification with 6 N HCl. They were isolated by filtration with suction, washing with water and drying over KOH and P_2O_5 in 80–90% yield.

		Calc.	Found
N-chloroacetyl-5-amino-isophthalic acid (III) $C_{10}H_8ClNO_5$ (257.6)	C	46.62	46.76
	H	3.13	3.11
	Cl	13.76	13.85
	N	5.44	5.20
N-bromoacetyl-5-amino-isophthalic acid (II) $C_{10}H_8BrNO_5$ (302.1)	C	39.76	39.91
	H	2.66	2.66
	N	4.64	4.56

Purity was checked by TLC on SI F-plates (Riedel) in butanol–acetic acid–water (4:1:1). NMR-data were in accordance with the structure.

2.2. *Cell suspensions*

Hyperdiploid EAT cells grown in female NMRI mice were used after the first in vitro passage of 14 h for all experiments. For further details see [2,8]. Viability of the cells was tested by staining with nigrosin according to Kaltenbach [9]. Protein determination was performed with the microbiuret method [10].

2.3. *Chemical and enzymatic tests*

The reactivity of BASA, BAAPA, CAAPA, IAc and IAA with Cys or GSH was tested by incubating 0.5 μmol of the thiol with 30 μmol of the alkyl halide in a total volume of 10 ml 0.15 M phosphate buffer pH 7.5. Determination of the SH content at time interval of 1 min was carried out by adding 1 ml of the mixture to 0.5 ml of carboxypyridine disulfide (2 μmol CPDS/ml). The absorbance at 344 nm of the resulting carboxypyridinethion [11] indicates free remaining SH groups of Cys or GSH. The inhibitory effect of the reagents on intracellular SH functions was determined by incubating intact EAT cells at 37°C in Hanks balanced salt solution (HBSS) containing the appropriate reagent. For concentrations and incubation time see tables. The determination of the non-protein thiol content was as described in [1] after removal of the excess reagents by twice washing the cells in HBSS and subsequent ultrasonic disruption. The activity of glyceraldehydephosphate dehydrogenase (GAPDH, EC 1.2.2.12) in sonicates was measured according to the method of Bergmeyer [12]. Measurement of the *p*-NPPase activity of intact cells was as described in [1].

3. Results and discussion

3.1. *Reaction with low molecular weight SH compounds*

The chemical reaction mechanism of BASA, BAAPA, CAAPA with thiols involves nucleophilic substitution by the SH group to give products containing stable S–C bonds, as described for many other haloalkyl derivatives. It was therefore of interest to obtain information on the reaction rate of the new reagents with SH compounds.

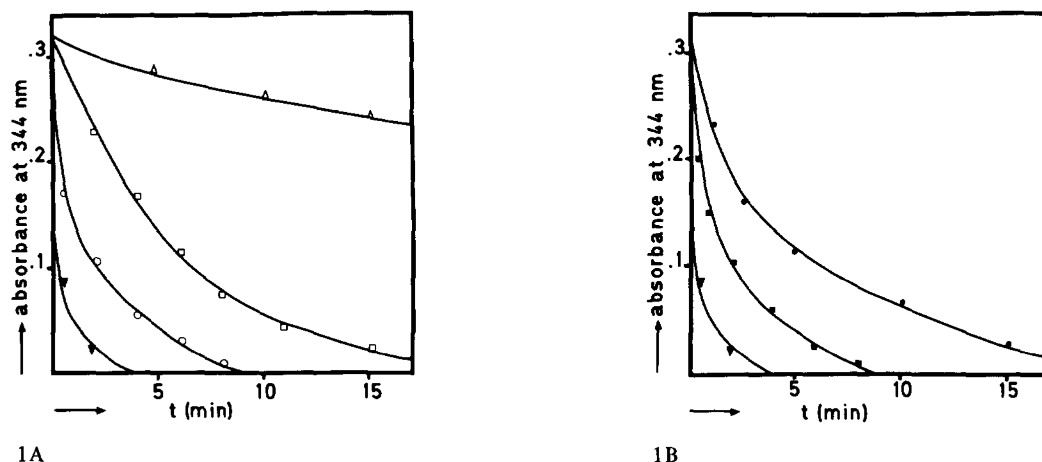

Fig.1. Reaction of haloalkyl derivatives with Cys and GSH (see methods 2.3.). (A): (△) CAAPA + Cys; (□) BAAPA + GSH; (○) BAAPA + Cys; (▾) BASA + Cys. (B): (●) IAc + Cys; (■) IAA + Cys; (▾) BASA + Cys.

It is evident from fig.1 that the reaction rate of the bromoacetyl derivatives compared with those of IAc and IAA is of the same order of magnitude, BASA reacting slightly more rapidly. The fact that the reaction of thiols with CAAPA proceeds rather slowly under physiological conditions is not surprising as the rate of reaction decreases progressively when bromine of alkyl halides is replaced by chlorine or fluorine [6]. Thus the chlorine derivative was not used for experiments with intact cells.

3.2. *Reaction with cellular non-protein SH*

The non-protein SH level of EAT cells which are cultivated in this laboratory averages 2.85 ± 0.25 nmol SH/10^6 cells. This value is in good agreement with those of Révész [13] who reported for different EAT cell lines 1.9–3.4 nmol SH/10^6 cells. On incubating EAT cells with thiol combining reagents, effects of different extent on the cellular SH level can be measured which undoubtedly depends upon the structure of the reagent [1,2]. The results of the treatment with BASA and BAAPA are summarized in table 1. Obviously there exists a considerable difference in behaviour of BASA and BAAPA and of IAc and IAA respectively. Using a lower concentration of 0.8 mM the iodine derivative penetrates the plasma membrane in a shorter time than the bromoacetyl derivative which seems to enter the cell interior at a significant rate only when the external concentration is above 2 mM. The present data do not give any information on the extent to which size, structure, type of halogen and charge characteristics contribute to this poor membrane penetrability. Table 1 also demonstrates a small but reproducible difference between the sulfonic and the carboxyl-group substituted derivatives. Similar results have been described for erythrocytes, the penetrability of *p*-chloromercuribenzene sulfonic acid being lower than that of *p*-chloromercuribenzoate [14].

3.3. *Effect on the GAPDH activity*

The activity of this glycolytic enzyme depends upon free SH groups [15] and is therefore very sensitive to thiol combining agents. Hence it is possible to decide whether and to what extent a reactive sulfhydryl blocking agent has passed the plasma membrane by measuring this enzymatic activity. The specific activity of the GAPDH of EAT cells which had been cultivated 4 h in the second in vitro passage was determined with 1,3-P_2-D-glycerate as substrate to be 1.5 ± 0.2 µmol/min·mg total cell protein. The influence of BASA, BAAPA and IAc on the activity after preincubation of the cells is summarized in table 2.

The question arises as to whether GAPDH is more reactive towards thiol combining agents than glutathione. To answer this one must recognize that part of the intracellular glutathione and other low molecular weight thiols are bound to protein, e.g. as mixed disulfides [16]. As the level of free glutathione decreases after exposure of the cells to alkylating

Table 1
Non-protein acid soluble SH level of EAT cells after preincubation with SH blocking reagents

Reagent	Concentration (mM)	Time of incubation (min)	non-protein SH % of control [± S.E.])
BASA	0.8	10	100 (2)
	1.6	10	98 (2)
	8.0	10	85 (4)
BAAPA	0.8	10	98 (3)
	1.6	10	97 (3)
	8.0	10	75 (4)
IAc	0.8	5	86 (2)
	1.6	5	78 (2)
IAA	0.8	5	64 (3)
	1.6	5	41 (2)

Table 2
Activity of GAPDH after preincubation with SH blocking reagents

Reagent	Concentration (mM)	Time of incubation (min)	GAPDH activity (% of control [± S.E.])
BASA	8.00	10	57 (4)
BAAPA	0.08	10	100 (4)
	0.80	10	90 (4)
	8.00	10	50 (3)
IAc	0.80	5	48 (2)

The results clearly demonstrate that BAAPA begins to penetrate at external concentration lower than 1 mM and not above 2 mM as might be concluded from the results in table 1.

Table 3
Effect of SH blocking reagents on the *p*-NPPase activity of intact EAT cells

Reagent	Concentration (mM)	*p*-Nitrophenylphosphatase activity (nmoles *p*NPP/10^7 cells·30 min [± S.E.])
none	–	153 (27)
BASA	0.1	109 (7)
BAAPA	0.1	106 (8)
IAc	0.1	113 (10)
IAA	0.1	114 (5)

reagents, this store could be mobilized. Since washing and sonication takes a few minutes it seems likely that the decrease in NPSH is not recorded in its entirety. By this interpretation, SH groups at the active center of GAPDH are not necessarily more reactive than the SH groups of low mol. wt. thiols. Rather the inhibitory effect of irreversible thiol combining agents as measured by the effect on GAPDH activity becomes evident in its entirety because the cells obviously cannot resupply the enzyme molecules within a few minutes. Our findings, independent, of interpretation, suggest that the entrance of thiol combining agents into the cytosol can be monitored reliably by measuring activities of cytosolic enzymes containing essential SH groups.

3.4. *Effect on the* p-*nitrophenylphosphatase activity*

We reported previously the inhibitory effect of different SH blocking reagents on the *p*-NPPase which we think is associated with the outer plasma membrane. In addition to our former investigation we studied the effect of BASA and BAAPA on the same system. The results compared with those of IAc and IAA are given in table 3.

The extent of inhibition (nearly 30%) is comparable to that observed with IAc and IAA. The SH groups on the cell surface, which must be in a reduced state for optimal phosphatase activity, are equally susceptible to each of the alkylating reagents tested. These results stand in contrast to those observed for mercurials and maleimides [1].

On the basis of the present results it follows that bromoacetylsulfanilic acid and bromoacetylamino-isophthalic acid can be regarded as very effective sulfhydryl alkylating reagents. Since chemical modification of membranes offers a useful probe with which to investigate structural and functional aspects of membrane organisation, both reagents could be a convenient tool in studying membrane phenomena, e.g. transport processes. Because of their limited membrane penetrability compared with iodoacetamide and iodoacetate these compounds seem to be advantageous for the study of experimental systems involving intact living cells. Whether the special characteristics of these reagents which are observed with EAT cells can be confirmed with other cells or cellular organelles remains to be investigated.

Acknowledgement

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