Biochemical Pharmacology, Vol. 37, No. 7, pp. 1245–1250, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

MECHANISMS OF CELL ASSOCIATION OF SOME NON-STEROIDAL ANTI-INFLAMMATORY DRUGS WITH ISOLATED LEUCOCYTES

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(*Received* 8 *October* 1986; *accepted* 1 *October* 1987)

Abstract—In the present study the degree and the mode of association of the radiolabelled drugs acetylsalicylic acid, sodium salicylate, and sodium benzoate with leucocytes were studied in view of the hypothesis that leucocytes are target cells for the anti-inflammatory activity of drugs. The overall association rate of acetylsalicylic acid is larger than that of sodium salicylate and sodium benzoate at 37°, but smaller at 4°. The ratio of the intracellular to the extracellular concentration varied between 1 and 2 for sodium salicylate and sodium benzoate, and between 3 and 6 for acetylsalicylic acid. The intracellular concentrations of these drugs were comparable in red blood cells and polymorphonuclear leucocytes, but lower in mononuclear leucocytes. The association of acetylsalicylic acid and sodium salicylate is markedly increased when the extracellular pH decreases. Lysis of cells decreases the association of acetylsalicylic acid and enhances the association of sodium salicylate and benzoate at 37° twofold. It is suggested that the association of these drugs with leucocytes comprises binding to the membrane and uptake of undissociated species. Phorbol myristate acetate extensively inhibits the intracellular concentration of acetylsalicylic acid, while this inflammatory stimulus tends to increase the intracellular concentration of sodium salicylate. The major metabolites of salicylate enhance cell association of acetylsalicylic acid and salicylic acid. In conclusion, these findings indicate that the tested benzoic acid-like drugs associate with leucocytes *in vitro* to some extent and that environmental differences, e.g. pH, lysed cells, inflammatory stimuli and metabolites, may determine *in vivo* the degree of accumulation.

Cellular pharmacokinetics is of eminent interest when a drug accumulates in a cell and when the cell is a therapeutic target of that drug. Non-steroidal anti-inflammatory drugs (NSAIDs)‡ are especially relevant to be examined in view of their suggested mode of action at the cellular level. The literature describing these mechanisms is extensive, and deals with inhibition of endogenous prostaglandin synthesis [1] and 15- and 11-lipoxygenase [2], inhibition of the migration of polymorphonuclear leucocytes (PMNs) and inhibition of lysosomal enzyme release from and superoxide anion radical generation by PMNs [3–7].

Furthermore, from the work of Inglot and Wolna [8], Famaey and Whitehouse [9] and MacGregor *et al.* [10], it appears that NSAIDs might have an effect on the cell membrane of erythrocytes and leucocytes. However, the molecular interaction between cells, e.g. leucocytes, and NSAIDs has not been studied intensively. Nevertheless, this aspect is especially relevant in view of the selective retention of NSAIDs by inflamed and acidic tissues *in vivo* [11, 12], the physiological accumulation of PMNs in inflamed areas in acute inflammation and the many actions of NSAIDs in relation to PMNs. Therefore, we undertook a series of *in vitro* studies to examine quantitatively whether a molecular affinity occurs between some benzoic acid-like compounds and cells, and furthermore which environmental factors influence this interaction. In a previous study [13] we reported a low but significant degree of molecular association of sodium salicylate (SA) with PMNs. In the present work we show the molecular affinity of benzoic acid (BA) and acetylsalicylic acid (ASA) besides SA to some types of blood cells and how this is influenced by the extracellular concentration, temperature, pH, type of the cells, metabolic activation of the cells, metabolites and the presence of human serum albumin (HSA).

‡ Abbreviations used: NSAIDs, non-steroidal anti-inflammatory drugs; PMNs, polymorphonuclear leucocytes; ASA, acetylsalicylic acid; SA, sodium salicylate; BA, benzoic acid; LDH, lactate dehydrogenase; RBCs, red blood cells; MNLs, mononuclear leucocytes; HSA, human serum albumin; SUA, *o*-hydroxyhippuric acid; GA, 2,5-dihydroxybenzoic acid; PMA, phorbol 12-myristate 13-acetate; CB, cytochalasin B; DIDS, 4,4′.diisothiocyano-2,2′-disulphonic acid stilbene disodium salt; DPBS, Dulbecco's phosphate-buffered saline; DMSO, dimethylsulfoxide.

MATERIALS AND METHODS

Cell isolation. Human polymorphonuclear leucocytes (PMNs) were purified from heparinized human blood by a previous described technique [13] employing sequential dextran sedimentation, Ficoll-Paque (sp.gr. 1.077) gradient centrifugation and Percoll (sp.gr. 1.098) gradient centrifugation (Pharmacia Fine Chemicals, Uppsala, Sweden). Final cell suspensions in Dulbecco's phosphate buffered saline

($20–30 \times 10^6$ cells/ml) with 0.1% w/v glucose, pH 7.4 (DPBS), contained more than 95% granulocytes and were at least 95% viable as judged by trypan blue exclusion and lactate dehydrogenase (LDH) measurement. Blood donors were healthy adult volunteers, who all gave informed consent.

Human red blood cells (RBCs) and mononuclear cells (MNLs) were isolated from heparinized peripheral blood as described before [14]. The final cell pellets were resuspended in DPBS ($20–30 \times 10^6$ cells/ml). Viability as tested by trypan blue exclusion was always greater than 95%.

The mononuclear cell fraction contained mostly lymphocytes (75–80%) and further monocytes (20–25%) and some granulocytes (0–3%), as determined by a microscopic count after staining of cytocentrifuge slides with May Grünwald–Giemsa and staining for nonspecific esterase [15]. A further purification of the MNL fraction was achieved by discontinuous density gradient centrifugation techniques according to the slightly modified method of De Boer *et al.* [16]: instead of resuspending the MNL fraction, after 30 min incubation at 37°, in Ficoll solution of sp.gr. 1.062 g/cm^3, a Percoll fraction of 1.063 g/cm^3 was used as suspending medium [17]. The purified cells were resuspended in DPBS supplemented with 0.1% w/v HSA. Purity was established by differential counting of 100 cells in cytocentrifuge preparations, stained with May Grünwald–Giemsa and for nonspecific esterase, and was about 42 and 98% for monocytes and lymphocytes, respectively. The viability was at least 95% for both blood preparations as measured by Trypan blue exclusion.

Ligand binding assays. Cellular association studies of ligands were performed by the silicone oil cushion method and the double centrifugation method, as previously described [13]. Radiolabelled ligand was added to the incubation medium at appropriate temperatures; at time 0 an aliquot of a purified cell suspension containing 3×10^6 cells was added. The total incubation volume was 600 μl. After incubation, at a certain temperature and for a certain period of time, the cells were separated from medium by centrifugation through the oil cushion for 5 min at 18,000 *g* in a Heraeus Hämofuge (Heraeus Christ, Osterode, F.R.G.). The radioactivity in the cell pellet was counted and the amount of cell associated drug was calculated.

In some experiments cells were lysed by rapid freezing followed by sonication. After this treatment microscopic analysis revealed no intact cells anymore. These lysed cells were used in the ligand binding assays to measure the degree of drug associated with membrane fragments.

The ligand binding assays in which the effect of pharmacological tools such as metabolic inhibitors or activators on the ligand–PMN interaction was studied, were performed in another sequence of additions, unless otherwise stated. The cells were preincubated with the pharmacological probes for 5 min at 20° after which the radiolabelled ligand was added.

Intracellular volume and drug measurement. Routinely the intracellular volume was measured as described before employing [^{3}H]H_2O and hydroxy-[^{14}C]methylinulin as markers [14]. Inulin is used as marker for the extracellular space. The intracellular water space could then be estimated by subtracting the hydroxy[^{14}C]methylinulin space from the [^{3}H]H_2O space. The inulin space was always less than 20% of the water space. The intracellular drug concentration can be estimated by dividing the amount of cell associated drug by the intracellular volume.

LDH determination. Release of cytoplasmic LDH was assayed by measuring the LDH catalysed reduction of pyruvate to lactate in the presence of NADH as was earlier described [14].

Composition of the incubation buffers for the pH experiments. The experiments were performed in 6–7 different incubation buffers with the standard composition (in mmol/l): NaCl 150.5, KCl 2.7, $CaCl_2.2H_2O$ 1.2, $MgCl_2.6H_2O$ 1.1, glucose.$1H_2O$ 5.6. The desired pH of the solution was obtained by adding 66 mmol/l of $Na_2HPO_4.2H_2O$ and KH_2PO_4 in appropriate proportions and was constant during the incubation at 37° (the osmolality of the solutions varied between 280–320 mOsm).

Data presentation. The cell association of the various compounds is defined as the total amount of nanomoles associated with 3×10^6 intact or lysed cells as a function of time or extracellular concentration. The intracellular drug concentration could by definition only be measured in experiments with intact cells and symbolizes the total amount of cell associated drug per intracellular volume unit. The cell/medium ratio of a drug expresses the ratio of the estimated intracellular drug concentration to the measured extracellular concentration of that drug in the incubation medium. All comparisons were made within incubation sets. The results are presented as mean values ± the standard deviation (SD). The statistical significance of differences was determined by analysis of variance followed by Student's *t*-test.

Reagents. HSA (essentially fatty acid free), phorbol 12-myristate 13-acetate (PMA), probenecid, monensin, cytochalasin B (CB), 2,4-dinitrophenol, and 4,4′-diisothiocyano-2,2′-disulphonic acid stilbene disodium salt (DIDS) were obtained from Sigma (St. Louis, MO). Sodium salicylate, acetylsalicylic acid, sodium benzoate, 2,5-dihydroxybenzoic acid (GA), *o*-hydroxyhippuric acid (SUA), g-strophanthin (ouabain) and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, F.R.G.).

All compounds were soluble under the incubation conditions applied and they produced no alteration in the pH of the medium. The incubation buffer was DPBS. The small amount of ethanol (maximal final concentration of 5% w/v) sometimes needed as solvent vehicle did not effect cell viability, as measured by LDH release and Trypan blue exclusion.

Spontaneous hydrolysis of ASA (0.1–2.1 mM) in DPBS was recorded by performing parallel experiments with nonlabelled ASA. ASA and SA levels were measured by chromatography [18]. The half-life of hydrolysis of ASA in DPBS (pH 7.4) at 37° was 24 ± 3 hr. Lowering of the incubation temperature from 37° to 4° resulted in about fourfold increase in the half-life of hydrolysis, in other words a fourfold decrease of the rate of hydrolysis. Obviously there

Table 1. Time course of the association of ASA (0.5 mM) with 3×10^6 intact PMNs at 37°

Time (min)	Cell associated ASA (nmol)	Intracellular conc. of ASA (mM)[a]	Cell/medium ratio[b]
1	0.23 ± 0.02	0.38 ± 0.03	0.7 ± 0.1*
5	0.44 ± 0.04	0.73 ± 0.06	1.4 ± 0.2*
10	0.49 ± 0.01	0.82 ± 0.02	1.6 ± 0.1*
20	0.51 ± 0.01	0.85 ± 0.02	1.7 ± 0.1*
60	0.51 ± 0.01	0.85 ± 0.02	1.7 ± 0.1*

Presented are the mean values ± SD of triplicate assays. One of three similar experiments is shown. Significant differences between intracellular and extracellular concentrations are designated by * ($P < 0.05$).

[a] The intracellular space was determined by performing binding assays with ^3H-H_2O and hydroxy(^{14}C)-methylinulin.

[b] The cell/medium ratio is expressed as the ratio of the estimated intracellular concentration to the measured extracellular concentration of ASA.

was a decrease in the rate of hydrolysis with decreasing incubation pH. From these results it was concluded that the degradation of ASA to SA at 37°, at pH 7.4 and maximally for 1 hr was 5% at most.

PMA (1.6 mM) and CB (10 mg/ml) were dissolved in DMSO and stored at −70° until use. Prior to the experiments these solutions were further diluted with DPBS. The final concentration of DMSO (< 0.02% v/v) had no detectable effect on any of the assays.

The radiolabelled compounds [^{14}C]salicylic acid (sp.act. 53.8 mCi/mmol), [carboxyl-^{14}C]acetylsalicylic acid (sp.act. 34.3 mCi/mmol), [^{14}C]benzoic acid (sp.act. 53.3 mCi/mmol), and [^3H]H_2O (sp.act. 0.25 μCi/mg) were purchased from New England Nuclear (Boston, MA). Hydroxy [^{14}C]methylinulin (sp.act. 1.32 μCi/mg) was obtained from The Radiochemical Centre Amersham (Buckinghamshire, U.K.). All the other chemicals were of analytical grade and obtained from Merck (Darmstadt, F.R.G.).

RESULTS

Characterization and viability of PMNs during incubation with benzoic acid derivates

During the incubation period of maximal 60 min with drug concentrations of 0–60 mM at 37°, the cell count and viability, as measured by Trypan blue exclusion, were 90% at least; after incubation the LDH release was measured immediately and appeared to be less than 11% of a positive control (the same number of sonicated PMNs in DPBS) [14].

Time- and concentration-dependent association

The initial rate of association of SA and BA was much greater than that of ASA. Kinetic data could not be determined accurately because of the high association rates and the rather long time required to terminate the reaction between PMNs and ligands (at least 1 min). This is not a major disadvantage if equilibrium drug levels are desired. As an example a typical time course of ASA association to PMNs is shown in Table 1. The extent of cellular association at steady state depends on the external concentration (Table 2). The overall association rate of ASA is much larger than that of SA and BA at 37°. SA and BA, but not ASA, show a decrease of the cell association when the extracellular concentration of the drugs increase, suggesting a saturable association mechanism.

Effect of cell type and cell intactness on the ligand association

The degree of cell association of salicylates with MNLs is about two times lower than the amount of cell associated salicylate with PMNs (40–50% in the presence of 0.1–2.1 mM ASA and 50–60% in the

Table 2. Concentration dependence of the association of ASA, SA and BA after 60 min incubation with 3×10^6 intact PMNs at 37°

Conc. of drug added	Cell/medium ratio[a]
0.1 mM ASA	3.9 ± 0.1**
0.5 mM ASA	3.8 ± 0.2**
1.2 mM ASA	4.9 ± 0.4**
2.1 mM ASA	5.9 ± 0.4**
0.8 mM SA	2.1 ± 0.2*
1.6 mM SA	1.9 ± 0.1*
2.5 mM SA	2.0 ± 0.2*
16.4 mM SA	1.5 ± 0.1*
0.5 mM BA	2.1 ± 0.1*
1.0 mM BA	1.9 ± 0.2*
2.0 mM BA	1.8 ± 0.1*
10.0 mM BA	1.6 ± 0.1*

The association was investigated at four different external concentrations, including also the recommended therapeutic concentration. The intracellular space was determined by performing binding assays with ^3H-H_2O and hydroxy(^{14}C)-methylinulin.

Presented are the mean values ± SD of triplicate assays. One of three similar experiments is shown.

Significant differences between intracellular and extracellular concentrations are designated by * ($P < 0.05$) and ** ($P < 0.01$).

[a] The cell/medium ratio is expressed as the ratio of the estimated intracellular concentration to the measured extracellular concentration of the drug.

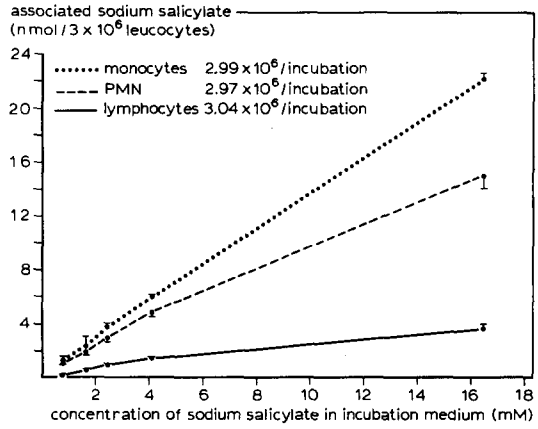

Fig. 1. Influence of the cell type on the concentration dependent association of SA. The mean values ± SD are plotted of triplicate assays at 37°. One of three similar experiments is shown. The incubation medium contained 0.1% w/v human serum albumin.

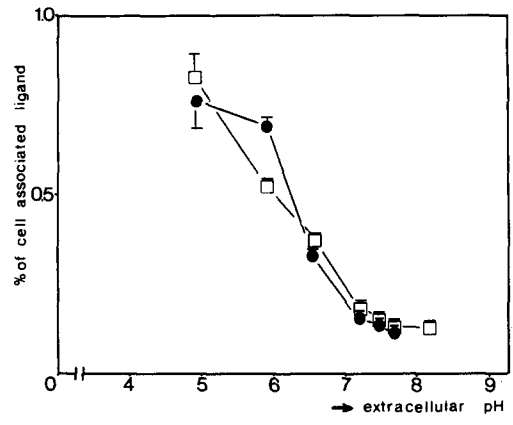

Fig. 2. Effect of the extracellular pH on the association of 0.5 mM ASA (●) and 4.1 mM SA (□) with 3×10^6 intact PMNs after 60 min incubation at 37°. Each point represents the mean and the SD of triplicate assays. One of three similar experiments is shown.

presence of 1.6–15.1 mM SA). RBCs incorporate less salicylate in comparison with PMNs in the same range of SA and ASA concentration (about 10%). On the contrary, when the intracellular concentrations of ASA and SA in PMNs, MNLs and RBCs are compared, it appears that the intracellular concentrations in RBCs approach the concentrations in PMNs, while the concentration in MNLs is much lower compared to PMNs (20–40% of the concentration in PMNs). Further differentiation of the heterogeneous MNLs results in a high degree of cell association of SA with monocytes and in a low degree of cell association with lymphocytes when compared to PMNs (Fig. 1). This low degree of salicylate association with lymphocytes causes the small degree of association of the MNL fraction, which mainly contained lymphocytes (see Materials and Methods). The intracellular volume of the cells (volume monocytes > PMNs > lymphocytes) appears to be the major determinant for the degree of cell association of the lipophilic drugs tested.

Table 3. Effect of cell intactness on the association of ASA, SA and BA at steady state at 37°

Conc. of drug added	Incubation of lysed PMNs at 37°
0.1 mM ASA	75 ± 15*
0.5 mM ASA	60 ± 4*
1.2 mM ASA	49 ± 5**
0.8 mM SA	167 ± 38*
1.6 mM SA	207 ± 31*
4.1 mM SA	187 ± 34*
0.5 mM BA	180 ± 20*
1.0 mM BA	187 ± 33*
2.0 mM BA	207 ± 18*

Lysed and intact cells (3×10^6) were separately incubated with different concentrations of drugs.

Results are expressed as % of a control incubation of 3×10^6 intact PMNs (lysed/intact × 100%) at 37° (N = 6). Significant differences between control and assay values are designated by * ($P < 0.05$) and ** ($P < 0.001$).

The data in Table 3 show at 37° a remarkable increase of the association of SA and BA with lysed PMNs in comparison with intact PMNs, while the affinity of ASA for lysed cells was lower than for intact cells.

Influence of the pH upon association of salicylates

Addition of 0.5 mM ASA or 4.1 mM SA to the incubation buffers had no influence on the adjusted pH. In all incubation media the viability of the PMNs was more than 95%. The association of ASA or SA with PMNs is markedly enhanced when the extracellular pH decreases (Fig. 2), suggesting passive nonionic diffusion as an important mechanism in the cell association.

Effect of phorbol 12-myristate 13-acetate on salicylate association with PMNs

The cell association of ASA is decreased by exposure of the PMNs to 0.13 μM phorbol 12-myristate 13-acetate (PMA). This concentration is sufficient to elicit release of superoxide anion radical generation by human PMNs without being toxic to the cells even in the presence of ASA (LDH release < 10%). This PMA concentration decreased ASA cell association significantly ($P < 0.001$) at an extracellular drug concentration of 0.5 mM ASA for 50% when calculated on basis of the intracellular concentration (Table 4). 0.13 μM PMA on the other hand tended to increase the intracellular concentration of SA at the anti-inflammatory concentrations of 1.5–2.1 mM SA. This increase is only 11–18%, but significant ($P < 0.05$). PMA cell stimulation caused an increase of the intracellular volume of 30 ± 11%.

Stimulation of salicylate cell association by its metabolites

The cell association of 0.5 mM ASA and 4.1 mM SA with PMNs is increased markedly in the presence of their metabolites salicyluric acid (SUA) and gen-

Table 4. Effect of pretreatment of the PMNs with PMA (0.13 μM) on the association of salicylates at a wide range of extracellular drug concentration at 37°

Extracellular drug concentration (mM)	ASA[a,b]	SA[a,b]
0.1	47 ± 2*	nd[c]
0.2	51 ± 3*	nd
0.5	52 ± 4*	123 ± 13
1.0	nd	125 ± 8
1.2	49 ± 3*	nd
1.5	nd	111 ± 2
2.1	51 ± 2*	118 ± 6

3×10^6 intact PMNs were treated with 0.13 μM PMA for 15 min at 37°. At time 0 radiolabelled ASA or SA was added and the incubation was continued for 60 min at 37°. Presented are the mean values ± SD of triplicate assays. One of three similar experiments is shown.

Significant differences between control and PMA values are designated by * ($P < 0.001$).

[a]Results are expressed as % of a control incubation (no pretreatment with PMA, but with all further conditions) and were calculated from:

$$\frac{\text{intracellular ASA or SA concentration in the presence of PMA}}{\text{intracellular ASA or SA concentration in the absence of PMA}} \times 100\%$$

[b]Intracellular volumes were 0.73 ± 0.03 μl/3.10^6 untreated PMNs and 0.95 ± 0.04 μl/3.10^6 PMA treated PMNs.

[c]Not determined.

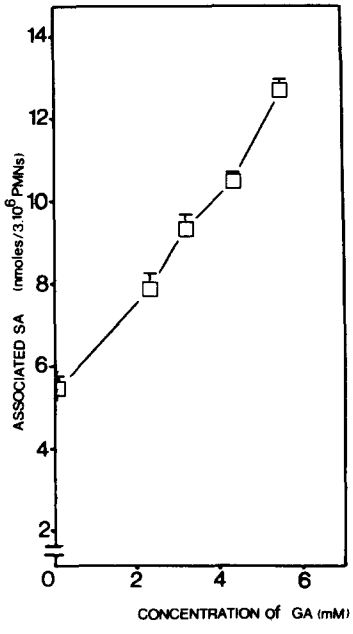

Fig. 3. Influence of various concentrations of the metabolite gentisic acid (GA) on the cell association of radiolabelled SA. The mean values are plotted of triplicate assays of 1 hr incubation at 37°. One of three similar experiments is shown.

tisic acid (GA) at a concentration range of 1–5 mM. This enhancement was proportional to the concentration of SUA or GA added and comprised 30–200% of the degree of cell association of an incubation without metabolite. The effect of GA on SA cell association is presented in Fig. 3. The LDH release in these experiments with metabolites and parent drugs was 6–11% after 60 min incubation at 37°. At higher metabolite concentrations (> 5 mM) cell lysis occurred.

In order to determine whether the influence of the metabolites on the cell association of ASA and SA was caused by changes in the membrane permeability, the effects of 5 μM DIDS on SA cell association in the presence of SUA or GA were studied. Stimulation of the cell association by SUA or GA was still observed, although the cell association of SA was significantly ($P < 0.05$) decreased by 10–30% in the presence of DIDS (data not shown).

Effect of various agents on the association of salicylates with PMNs

In order to determine whether the degree of cell association of ASA or SA was dependent on changes in cell energy, experiments were performed with some metabolic inhibitors and pharmacologic probes, whose mode of action is well documented. None of the compounds (ouabain, 2,4-dinitrophenol, monensin, cytochalasin B) used inhibits or stimulates cell association except of probenecid (2.3 mM) which stimulates cell association of ASA and SA.

DISCUSSION

In this paper we make an attempt to identify several factors that determine the association of ASA-like drugs with PMNs.

The time course of association is characterized by a very rapid (SA and BA) or slower (ASA) initial rate at 37°. The cell to medium concentration ratio estimated for the net associated component was significantly ($P < 0.01$) higher with ASA, which gave ratios from 3.8 to 5.9, than with SA and BA, which gave a rather constant value between 1.5 and 2.1, despite wide variations in the initial concentration. In contrast to ASA, however, SA and BA have significantly ($P < 0.05$) higher cell/medium ratios at low (< 1 mM) than at high (≥ 10 mM) drug concentrations (Table 2) suggesting saturable association mechanism.

From the experiments with intact and lysed cells it appears that the intact cell membrane promotes the association of only ASA with PMNs at 37° (cell association lysed < intact) ($P < 0.05$). The association of SA and BA at 37° with lysed cells is significantly higher than with intact cells ($P < 0.05$) indicating intracellular association sites for these compounds.

The degree of cell associated salicylate is largely increased at low pH, which indicates that the unionized species accumulates more easily in the PMNs. Since ASA and SA are weakly acidic drugs with pK_a values of 3.5 and 3.0, respectively, it is not surprising that their cell association is pH dependent. Therefore, cell association of these compounds is a diffusion-limited process governed by the physico-

chemical properties of the drug and the nature of the cell membrane.

Association of salicylates was studied in various cell types and appears to differ among cell types under similar conditions of incubation. The intracellular volume determines the amount of total associated salicylate per number of cells. However, the intracellular concentrations of salicylates are equal in PMNs and RBCs, but smaller in the non-purified MNL fraction. Our data with lysed cells in contrast suggest that the degree of cell association of ASA-like compounds not only is dependent on diffusion, but also on association sites in the cytosol or on the membrane.

Treatment of PMNs with PMA results in an extensive decrease of the intracellular concentration of ASA and a small enhancement of the intracellular concentration of SA. In contrast to diclofenac [19], no inhibition of high affinity binding sites of chemotactic peptide by ASA and SA was found (data not shown). The volume expansion of the cells after stimulation, which was recently suggested by Berkow and Baehner [20], was confirmed by our experiments. The PMA effect on ASA and SA cell association cannot be explained adequately, however, in terms of cell expansion. The mechanisms of interaction of the inflammatory stimulus PMA with ASA and SA remain to be solved.

The association of ASA and SA with PMNs changes when these NSAIDs are incubated in the presence of their major metabolites. Oxidative conversion and glycine conjugation of SA lead to gentisic acid (GA) and salicyluric acid (SUA), respectively. Both these metabolites enhance the cell association of ASA and SA. Cell lysis and alteration of the pH of the incubation medium are excluded as explanations for the enhanced association. The enhancement of the ASA and SA cell association by GA and SUA could be explained by an increase of the cell membrane permeability for these drugs or by an interference with the efflux pathway from the cell interior. Nishihata *et al.* [21] observed uptake of the ionized form of SA in the erythrocyte, which could be inhibited by the anion channel blocker DIDS. They suggest that *o*-hydroxybenzoates alter membrane permeability thus permitting low lipophilic compounds to be taken up across a membrane barrier and that DIDS blocks the transport of orthoisomers of benzoic acid [21–23]. Exposure to DIDS however did not reduce the enhanced ASA or SA cell association in the presence or absence of SUA or GA.

In the presence of probenecid, a parasubstituted benzoic acid derivative and well-known as an anion transport inhibitor, the cell association of ASA and SA is markedly enhanced possibly by inhibition of a facilitated diffusion of ASA out of the PMNs. The pH of the incubation medium was not influenced by addition of probenecid.

The failure to inhibit cell association of ASA and SA by metabolic inhibitors, confirms that no specific energy-dependent mechanisms are involved in the process of cell association. These benzoic acid derivatives associate with PMNs by (facilitated) diffusion of the unionized species across the lipid interior of the membrane and by association with the outer membrane and/or cytosolic matrix.

In conclusion, our results suggest that the observed selective localization of acidic NSAIDs in certain (acidic) body compartments such as inflamed areas [12, 24] cannot be explained at all by assuming that the leucocytes act as transport vehicles for ASA-like drugs in blood [13, 25], since only low affinity association was found. The differential effects of PMA on ASA and SA cell association need thorough study in future. Finally, the interference of metabolites on ASA and SA cell association may account for higher intracellular drug concentrations *in vivo*, but needs further study.

Acknowledgements—The assistance of Mr Jan A. M. Huisman and Mrs Lilibeth Triebels is gratefully acknowledged.

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