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BINDING OF THE MEMBRANE ACTIVE DRUGS TO BOVINE SERUM ALBUMIN AND HUMAN ERYTHROCYTE MEMBRANES

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Abstract—The binding of 14 non-steroidal anti-inflammatory drugs, 3 beta-adrenolytics and 8 miscellaneous drugs to bovine serum albumin (BSA) and human erythrocyte membranes (HEM) was investigated by using 8-anilino-1-naphthalene sulphonate (ANS) as a fluorescent probe. Anionic drugs which are known to protect proteins against denaturation strongly quench the fluorescence of the ANS–BSA complex, and to a lesser extent the fluorescence of the ANS–HEM complex. None of cationic or nonionic drugs tested so far are able to displace ANS from the ANS–BSA complex but some of them, known as "membrane active" agents, intensify the fluorescence of the ANS–HEM complex. Within the group of non-steroidal anti-inflammatory drugs only those drugs which are inhibitors of prostaglandin biosynthesis have been found to quench the fluorescence of the complexes of ANS with BSA or HEM. Both anionic and cationic "membrane active" drugs protect erythrocytes against hypotonic hemolysis but the mechanisms of this effect are different.

Non-steroidal anti-inflammatory drugs protect proteins against denaturation due to heat [1, 2], u.v.-irradiation [2] or chemical agents [2]. Anti-inflammatory drugs also prevent hypotonic hemolysis, but they share this property with beta-adrenolytics [6], local anaesthetics [7], tranquilizers [7, 8] and antihistaminics [8]. These drugs being cationic [6–9], anionic [4, 5, 9] or nonionic [5, 9] belong to different chemical groups and sometimes they are named "membrane active" agents [6]. Stabilization of the erythrocyte membrane against hemolysis by "membrane active" drugs may involve their binding to the membrane but the intimate mechanism of this effect remains unknown [4]. The aim of this work was to compare the binding of several "membrane active" drugs to albumin with the binding of these drugs to erythrocyte membranes, using a fluorescent probe 8-anilino-1-naphthalene sulphonate.

MATERIAL AND METHODS

The following substances were used: bovine serum albumin (Cohn fraction V) produced by Polish Serum and Vaccines Works (dialyzed and lyophilized 95–99% albumin); 8-anilino-1-naphthalene sulphonic acid ammonium salt (ANS) kindly supplied by Sigma Chemical Company; aspirin, Polfa, Poland; phenylbutazone (Butapyrazol), Polfa, Poland; indomethacin (Metindol), Polfa, Poland; niflumic acid, synthetized by the Institute of Organic Chemistry, Warszawa, Poland; flufenamic acid, kindly supplied by Parke Davis, U.S.A.; fenoprofen, kindly supplied by the Eli Lilly Company U.S.A.; naproxen, kindly supplied by Syntex, U.S.A.; mefenamic acid (Ponstan), kindly supplied by Parke Davis, U.S.A.; meclofenamic acid (Meclofenemate), kindly supplied by Parke Davis, U.S.A.; chloroquine (Arechin diphosphate), Polfa, Poland; isopyrine, Polfa, Poland; dipyrone (Pyralgin), Polfa, Poland; benzydamine hydrochloride (Benalgin), Polfa, Poland; aminophenazone (Pyramidon), Polfa, Poland; tetradecanoic acid (Myristic acid), Koch Light, England; sodium laurylsulphate, British Drug Houses Ltd., England; *p*-bromphenylanthranilic acid, synthetized in the Department of Pharmacology, Polish Academy of Sciences, Kraków, Poland; propranolol hydrochloride, Polfa, Poland; practolol hydrochloride, Polfa, Poland; pindolol (LB-46), Sandoz, Switzerland; chloropromazine hydrochloride, Polfa, Poland; promethazine hydrochloride, Polfa, Poland; quinidine sulphate, Polfa, Poland; procaine hydrochloride, Polfa, Poland.

Binding of 8-anilino-1-naphthalene sulphonate (ANS) to bovine serum albumin (BSA). The solutions of BSA (0·66 mg/ml), ANS (0·04 mM) and drugs (0·01–10 mM) were made up in 100 mM phosphate buffer pH 7. Acidic drugs were converted into sodium salts in 100 mM sodium hydroxide. BSA was incubated at room temperature for 10 min in the presence of a drug. ANS was added and the fluorescence was measured. The relative fluorescence was expressed as a percentage of the fluorescence of a control solution which contained no drug. The concentration of drug which quenches the relative fluorescence by 50% (IC_{50}) was calculated graphically from the line plotted for percentage of quenching of fluorescence versus log drug.

Binding of ANS to human erythrocyte membranes (HEM). HEM were obtained by the method described by Dodge *et al.* [10] except that "ghosts" were not lyophilized but suspended in 100 mM phosphate buffer pH 7·4, stored at 4° and used for experiments within 3 days. The protein content of HEM was estimated by

Lowry's method [11]. The suspension of HEM in buffer (0·08 mg of protein per ml) was incubated with 0·1–5 mM drug solution at room temperature for 10 min and ANS was then added (final concn 1 mM). Fluorescence was immediately measured against the solution containing no drug. In the case of compounds which increased fluorescence the concentration of drug which caused a double increase in the fluorescence (EC_{200}) was calculated.

Measurement of fluorescence. A Baird atomic spectrofluorometer was used to measure the fluorescence intensity. The excitation and fluorescence wavelengths were 380 nm and 472 nm, respectively (both uncorrected).

RESULTS

None of the tested compounds changed the fluorescence spectrum of solutions containing ANS–BSA or ANS–HEM complexes. Some of the tested compounds made ANS solutions fluorescent in the absence of protein (e.g. meclofenamic acid, mefenamic acid). In this case the values of the relative fluorescence obtained in the absence of protein have been subtracted from the relative fluorescence measured in the presence of protein.

The fluorescence of the ANS–BSA complex was quenched only by anionic drugs (Table 1) while all nonionic and cationic compounds were inactive at concentrations up to 10 mM.

Table 2 shows the influence of the compounds tested at a concentration of 1 mM on the fluorescence of the complexes of ANS with HEM or BSA. All anionic compounds, aspirin excepted, quenched the fluorescence of the ANS HEM complex. Not all the cationic compounds (chloroquine, quinidine, procaine and practolol) and none of the nonionic compounds influenced the fluorescence of the ANS–HEM complex. The remaining cationic compounds increased the fluorescence of this complex. These compounds have been tested at higher concentrations to calculate EC_{200}. The EC_{200} values were: 0·12 mM for chloropromazine, 0·6 mM for promethazine, 1·1 mM for benzydamine and about 2·5 mM for propranolol. The cationic compounds which did not increase the fluorescence of the ANS–HEM complex when tested at a concentration of 1 mM were also tested at a concentration of 5 mM, in which case an increase in fluorescence was observed only for quinidine (+30%) and pindolol (+50%), while procaine, practolol and chloroquine remained inactive.

DISCUSSION

Binding of a drug to the hydrophobic sites of albumin, which are detected by ANS, depends on the physico-chemical character of that drug. None of the

Table 1. Quenching of the fluorescence of anilino-naphthalene sulphonate (ANS) (0·04 mM) with bovine serum albumin (0·66 mg/ml) in the presence of drugs

Compound	Physico-chemical character	Clinical use	IC_{50} (mM)
Aspirin	anionic	anti-inflammatory	5·6
Phenylbutazone	anionic	anti-inflammatory	1·0
Indomethacin	anionic	anti-inflammatory	0·14
Meclofenamic acid	anionic	anti-inflammatory	0·23
Mefenamic acid	anionic	anti-inflammatory	0·16
Niflumic acid	anionic	anti-inflammatory	0·16
Flufenamic acid	anionic	anti-inflammatory	0·08
Fenoprofen	anionic	anti-inflammatory	0·5
Naproxen	anionic	anti-inflammatory	10·0
Dipyrone	nonionic	anti-inflammatory	>10
Aminophenazone	nonionic	anti-inflammatory	>10
Chloroquine	cationic	anti-inflammatory	>10
Izopyrine	nonionic	anti-inflammatory	>10
Benzydamine	cationic	anti-inflammatory	—*
Sodium lauryl sulfate	anionic	not used	0·1
Miristic acid	anionic	not used	1·0
p-bromphenylanthranilic acid	anionic	not used	0·06
Quinidine	cationic	anti-arrhythmic	>10
Procaine	cationic	local-anaesthetic	>10
Propranolol	cationic	beta-adrenolytic	>10
Practolol	cationic	beta-adrenolytic	>10
Pindolol	cationic	beta-adrenolytic	>10
Chloropromazine	cationic	neuroleptic	>10
Promethazine	cationic	antihistaminic	>10

* Not determined because of the strong fluorescence of the blank sample.

IC_{50} values were calculated graphically from the plot of the decrease in fluorescence against log concentration of a drug.

Table 2. Per cent change in the intensity of the fluorescence of the complex of anilino-naphthalene sulphonate with bovine serum albumin (ANS–BSA) or the complex of anilino-naphthalene sulphonate with human erythrocyte membranes (ANS–HEM) induced by drugs at a concentration of 1 mM

Drug	ANS–BSA	ANS–HEM
Chloropromazine	0	+540
Promethazine	0	+130
Benzydamine	0	+50
Propranolol	0	+34
Pindolol	0	+4
Aspirin	−22	0
Chloroquine	0	0
Dipyrone	0	0
Izopyrine	0	0
Practolol	0	0
Quinidine	0	0
Procaine	0	0
Naproxen	−39	−13
Aminophenazone	0	−17
Fenoprofen	−60	−32
Phenylbutazone	−50	−37
Niflumate	−80	−60
Meclofenamate	−70	—
Flufenamate	−80	−70
Miristate	−50	−80
Indomethacin	−100	−90
Lauryl sulphate	−90	−90

cationic compounds tested so far displace ANS from albumin even when the concentration ratio of drug to ANS is 250. Binding of organic anions to albumin may depend on their ability to involve hydrophobic forces in the process of binding [12–14]. The order of potency of anti-inflammatory drugs to displace ANS from BSA is parallel to their order of potency to displace danzylamide [15], sulphonamides [16] or thiopenthal [17] from albumins, and to protect serum albumin against denaturation [1, 2]. Albumin is not the only protein which binds organic anions. The fibrinolytic activity of several acidic anti-inflammatory drugs has been described [18]. Antiviral activity [19], antihemolytic activity [3–5], protection against heat coagulation [1, 2] and inhibition of a number of enzymes [20] are common features of acidic non-steroidal anti-inflammatory drugs. These compounds unlike steroidal anti-inflammatory drugs also inhibit prostaglandin synthetase [21] and this may explain the mechanism of their pharmacological action [22]. According to our results [23] several anionic anti-inflammatory drugs inhibit prostaglandin synthetase activity. In the present experiments we found that the same drugs displace ANS from BSA (first nine compounds in Table 1). Cationic anti-inflammatory drugs, tested in our laboratory (dipyrone, aminophrenazone, izopyrine, benzydamine and chloroquine) were unable to inhibit biosynthesis of prostaglandin. However, there is also some data indicating that cationic anti-inflammatory drugs, e.g. [24] compound L 8027 (2-isopropyl-3-(2′-pyridyl-oxo)-indole), are inhibitors of prostaglandin synthetase.* None of the cationic compounds tested by us and listed in Table 1 quenched the fluorescence of the ANS–BSA complex.

Binding of ANS to the lipid–protein interphase of biological membranes produces a fluorescent complex [25–27]. The fluorescence of this complex may be increased or decreased by drugs. Wiethold *et al.* [6] have found that only those beta-adrenolytic drugs which possess "unspecific membrane activity" may increase the number of binding sites for ANS in human erythrocyte membranes and thus increase the fluorescence of the complex. The authors suggest that accumulation of cationic beta-adrenolytic drugs within the membrane leads to enhanced interaction of anionic ANS with the drug–membrane complex. It seems however that chemical structure rather than the mere negative charge of a drug determines its binding to the membrane, because a number of cationic drugs (e.g. practolol, procaine, chloroquine) do not increase the fluorescence of the ANS–HEM complex. We have confirmed the conclusion of Wiethold *et al.* [6] that the increase in fluorescence of the ANS–HEM complex may be a measure of the quinidine-like or local anaesthetic activity of beta-adrenolytic drugs. However it is difficult to explain the ineffectiveness of procaine itself to increase the fluorescence of the complex. The fact that positively charged compounds never increased the fluorescence of the ANS–BSA complex favours the assumption that they are bound to the lipid–protein interphase of the membrane, rather than to the membrane protein [28].

Binding of drugs to the erythrocyte cell membrane correlates with their influence on erythrocyte stability in hypotonic solutions. Drugs which are not bound to the membrane, e.g. aminophenazone, also have no antihemolytic activity. The only exception is chloroquine which prevents or facilitates hemolysis depending on its concentration [3] but is not bound to the cell membrane (Table 2). Drugs which quench the fluorescence of the ANS–HEM complex, e.g. anionic anti-inflammatory drugs (Table 2), prevent or facilitate hemolysis depending on the temperature [29] and their antihemolytic activity is increased at low pH [3]. Local anaesthetics [7], tranquilizers [7, 8] and antihistaminics [8] have been found to increase the fluorescence of the ANS–HEM complex and at the same time they prevent hemolysis independently of temperature [28] and this activity is enhanced at high pH [3]. Thus quenching or enhancing of the ANS–HEM fluorescence by a drug strongly indicates its type of antihemolytic activity.

* Compound L 8027 quenches the fluorescence of ANS with BSA in 50% at a concn of 0·65 mM.

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