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# Biothermodynamic characterization of erythrocyte hemolysis induced by phenothiazine derivatives and anti-inflammatory drugs

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Many ionic drugs which are distributed and/or bound to human blood cells frequently induce erythrocyte hemolysis with different rate and extent at high drug concentrations [1–4]. Drug-induced hemolysis has been recognized as being classified into two main categories: direct interaction of the drug with the erythrocyte membranes [4] and indirect disruption of the integrity in the cellular metabolism produced by the drug [5]. It is well known that phenothiazine derivatives and non-steroidal anti-inflammatory drugs induce hemolysis where the chief mechanism is the direct interactions with the membranes, whereby the hemolytic activity is dependent on both binding to, and membrane-perturbating ability of, the erythrocytes [6–9]. However, there has been, as yet, no satisfactory elucidation of the differences in hemolytic action between these drugs, nor has the precise mechanism by which drug-induced hemolysis is brought about at high concentrations been made known.

Recently we reported the usefulness of microcalorimetry for the study of drug interaction in the blood system [10–12]. The thermodynamic parameters in the drug reaction process may include structural contributions arising from different molecular events and changes in the aggregation state of either reactant. In the present study, the biothermodynamic characteristics of the erythrocyte hemolysis induced by ionic drugs, phenothiazine derivatives as cationics and non-steroidal anti-inflammatory drugs as anionics, were observed, in an attempt to define the mechanism of hemolysis.

## *Materials and methods*

Fluphenazine dimaleate (FPZ), perphenazine dimaleate (PPZ), trifluoperazine dimaleate (FPRZ), prochlorperazine dimaleate (CPRZ) and perazine dimaleate (PRZ) were obtained from Yoshitomi Pharm. Ind. Ltd (Osaka, Japan). Flufenamic acid (FA), mefenamic acid (MA), ibuprofen (IP) and indomethacin (IM) were purchased from the Sigma Chemical Co. (St Louis, MO), and flurbiprofen (FP) was a gift from Kaken Pharm. Co. Ltd (Tokyo, Japan). Stock solutions (0.2 M) of IM, FA and MA were prepared in dimethyl sulfoxide and diluted with phosphate-buffered isotonic saline (10 mM sodium phosphate, 140.5 mM NaCl, pH 7.4) (PBS). Other drugs were directly dissolved in PBS. Human erythrocytes were supplied by the Red Cross Blood Centre (Fukuoka, Japan) and washed three times with PBS by centrifugation at 1000 *g* for 10 min and then resuspended in the same buffer as a stock solution. Before each use, the erythrocytes were washed with PBS until the supernatant fraction was clear and colorless, in order to obtain packed cells with a 100% erythrocyte concentration. The number of erythrocytes in each experimental suspension was measured by a Coulter Counter model TA-2 (Hialeah, FL).

In the experiments of drug-induced hemolysis, a drug solution was added to a 2% (v/v) erythrocyte suspension in PBS by microsyringe at a drug concentration between $10^{-5}$ and $10^{-2}$ M. The mixture was incubated for 90 min at 37° and then centrifuged at 1000 *g* for 10 min. The supernatant fraction was separated, and the absorbance $E^{1cm}_{543nm}$ of hemoglobin released from the erythrocytes was determined. The percentage of hemolysis is expressed by the ratio of absorbance at 543 nm against the complete hemolysis of the erythrocytes in water.

Calorimetric measurements were carried out at 37.0 ± 0.05° using a differential flow-microcalorimeter [12] and a Rikadenki chart recorder (Tokyo, Japan). The reaction solutions were introduced into the calorimeter through Tigon tubin with a four-channeled peristaltic pump (Gilson minipuls 2, Villers-Le-Bel, France). The procedures used in the flow experiments have been described in detail elsewhere [12]. For the heat effect on drug-induced hemolysis, the dilution heat of erythrocytes was measured continuously. A calorimetric record of the heat produced by erythrocyte hemolysis during incubation of a 4% (v/v) erythrocyte suspension with FPRZ is shown in Fig. 1. The enthalpy change of the hemoglobin released from one red cell ($\Delta H_{hemol}$) was calculated from a steady-state level of the signal representing the complete hemolysis by use of the following equation:

$$\Delta Q = \Delta H_{hemol} \cdot Fr \cdot [E],$$

where $\Delta Q$, $Fr$ and $[E]$ represent the heat effect, the flow rate, and the number of erythrocyte cells respectively.

## *Results and discussion*

Figure 2 shows the calorimetric (upper) and percent hemolysis (bottom) profiles during incubation of 4% (v/v) erythrocyte suspensions at various concentrations of PPZ and FA. As shown in the case of PPZ (Fig. 2A) and FPRZ (Fig. 1), the phenothiazines brought about immediate hemolysis upon drug exposure, and the heat of hemolysis was increased endothermically with an increased degree of hemolysis. On the other hand, a different pattern was observ-

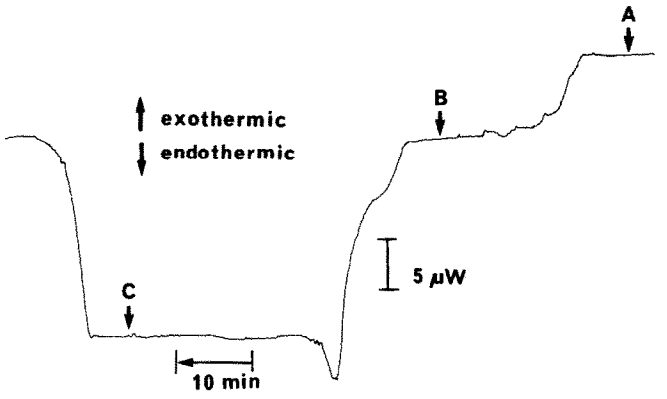


Fig. 1. Calorimetric record of heat produced during erythrocyte hemolysis induced by FPRZ at 37°. Initial base line was established by PBS solution which was pumped through two flow lines into the calorimeter at a flow rate of 0.157 ± 0.002 ml/min. At point A, 4% (v/v) erythrocyte suspension was allowed to flow into the calorimeter via one of the flow lines, and then the second base line which was dependent upon the heat of dilution of intact erythrocytes was newly established. After 20 min, 2 mM FPRZ was added to the erythrocyte suspension (B), and then the heat produced in hemolysis induced by FPRZ began to be recorded. A steady-state level of the calorimetric signal (approximately 15 μW below the second baseline) corresonded to the complete hemolysis induced by FPRZ. At point C, a new erythrocyte suspension was allowed to flow instead of developing hemolyzed erythrocyte suspension to reestablish the initial baseline. The concentrations of FPRZ and erythrocytes in the suspension were estimated to be 0.314 mM and 3.37%, respectively. The dilution heat of drugs was instrumentally subtracted by the differential calorimetric method [12].

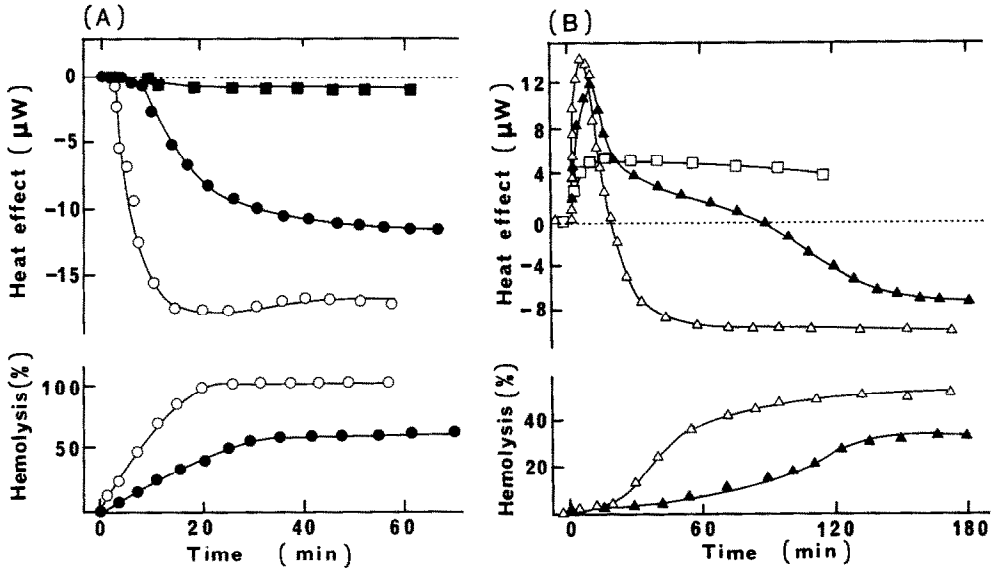


Fig. 2. Time courses of heat effect (upper) and percent hemolysis (bottom) during human erythrocyte hemolysis induced by PPZ (A) and FA (B) at 37°. Human erythrocytes in 4% (v/v) suspension were incubated with 0.5 mM (○), 0.2 mM (●) and 0.1 mM (■) PPZ and 1.2 mM (△), 0.8 mM (▲) and 0.1 mM (□) FA. Percent hemolysis is expressed as the ratio of the absorbance at 543 nm in the final calorimetric solution to the absorbance after the complete hemolysis in water.

able in hemolysis induced by FA, an anti-inflammatory drug (Fig. 2B). The calorimetric profiles of hemolysis at the higher concentrations of FA (0.8 and 1.2 mM) were characterized by two stages: the heat effect was produced first exothermically and then reversed to an endothermic process. The percentage of hemolysis increased gradually following a lag phase for about 20 min. At a lower concentration of FA (0.1 mM), where no significant hemolysis occurred, the heat effect produced was exothermic. In all the cases of hemolytic patterns, there was a significant correlation between the heat effect and the degree of hemolysis, indicating that the heat produced endothermically

Table 1. Hemolytic concentrations of drugs and the enthalpy changes of hemoglobin released from one red cell

| Drugs | $C_1$ (mM) | $C_{50}$ (mM) | $C_{100}$ (mM) | $\Delta H_{hemol}$ (pJ/cell) |
|---|---|---|---|---|
| Fluphenazine | 0.08 | 0.09 | 0.15 | 20.4 ± 1.4 |
| Perphenazine | 0.15 | 0.19 | 0.4 | 19.7 ± 1.5 |
| Trifluoperazine | 0.10 | 0.13 | 0.2 | 19.6 ± 1.9 |
| Prochlorperazine | 0.15 | 0.22 | 0.5 | 21.9 ± 1.2 |
| Perazine | 0.40 | 0.59 | 1.0 | 19.4 ± 2.1 |
| Flufenamic acid | 0.70 | 1.45 | 2.3 | 19.2 ± 1.8 |
| Mefenamic acid | 1.7 | 3.4 | 6.8 | 19.0 ± 2.0 |
| Ibuprofen | 3.4 | 7.2 | 10.1 | 18.5 ± 3.2 |
| Flurbiprofen | 1.4 | 2.2 | 4.0 | 20.9 ± 1.6 |
| Indomethacin | 1.0 | 1.95 | 3.8 | 21.1 ± 2.1 |

Abbreviations: $C_1$, the initial concentration inducing hemolysis; $C_{50}$ and $C_{100}$, the concentration inducing 50% and complete hemolysis respectively.

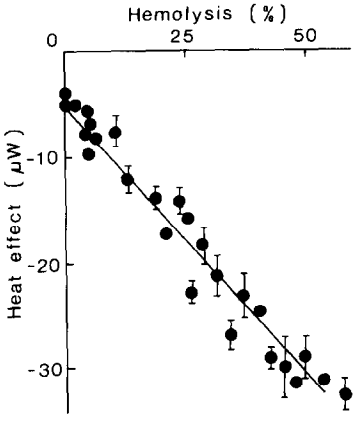


Fig. 3. Relationship between heat effect and percent hemolysis for osmotic hemolysis. Human erythrocyte suspensions of 10% (v/v) in phosphate-buffered hypotonic saline, pH 7.4, were incubated for 60 min at 37° and then centrifuged at 1000 *g* for 10 min. The heat of dilution by the same buffer and the absorbance $E^{1cm}_{543nm}$ of the supernatant fractions were measured under the same conditions as shown in Figs 1 and 2.

by hemolysis was related to the free hemoglobin released.

Table 1 summarizes the hemolytic concentrations of drugs and their $\Delta H_{hemol}$. Comparing the apparent hemolytic action of drugs, non-steroidal anti-inflammatory drugs caused hemolysis at a higher concentration than phenothiazines. The hemolytic activities, which represented reciprocals of the drug concentration inducing hemolysis, decreased in the order of FPZ > FPRZ > PPZ → CPRZ > PRZ and FA > IM > FP > MA > IP. However, the $\Delta H_{hemol}$ of all drugs was nearly equal, and the mean value was estimated to be 20.0 ± 1.0 pJ/cell.

To investigate whether the endothermic heat was related to the erythrocyte hemolysis or to the drug interaction with erythrocytes, the heat effect by osmotic hemolysis was measured under the same conditions. Figure 3 shows the correlation between the heat effect and the percentage of osmotic hemolysis, with a correlation coefficient of 0.982. The dilution enthalpies of an intact erythrocyte cell and a hemolyzed cell were found to be about 4.0 and 22.0 pJ/cell respectively. The latter value agreed with the mean value of $\Delta H_{hemol}$ estimated from drug-induced hemolysis. It has been suggested that the difference in the calorimetric profiles for hemolysis induced by anionic and cationic drugs thermodynamically may reflect some extent in the difference of the mechanisms. Since, however, the data are still insufficient and the investigation is still in progress, the present report should be regarded as only preliminary.

In summary, the heat effect of hemolysis was endothermic and related in proportion to the quantity of free hemoglobin released from erythrocyte cells rather than hemolytic activities of drugs, indicating that $\Delta H_{hemol}$ may be an important index of the hemolysis; $\Delta H_{hemol}$ = 20.0 ± 1.0 pJ/cell.

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