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ERYTHROCYTE MEMBRANE STABILIZATION AND PROTEIN BINDING OF SOME ANTI-INFLAMMATORY DRUGS AND OF DEOXYCHOLIC ACID

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Abstract—The stabilizing activity of indomethacin and phenylbutazone on red cells in aqueous solutions has been confirmed. The same activity, together with a hemolytic effect at higher concentrations, has been observed for deoxycholic acid and benzydamine. The activity of benzydamine and phenylbutazone remain practically unchanged in the presence of serum, whereas with indomethacin and deoxycholic acid, serum produced a sharp decrease in activity. The latter results were found to correlate with the different capacity of the substances to bind serum proteins.

SEVERAL drugs belonging to tranquillizers, antihistamines, local anesthetics and non-steroid anti-inflammatories protect red blood cells against hypotonic hemolysis and produce hemolysis at high concentrations.[1–3] The latter effects have been hitherto connected to the action mechanism of such drugs, taking into consideration that they might produce a change into the selective permeability of the cell or cell organelles membrane. Little attention has been paid, however, to the fact that the concentrations found effective *in vitro* by using saline solutions are closely similar to those actually achieved in human serum with therapeutic doses. The possibility existed, therefore, that specific changes into red blood cells might be produced in humans. Testing this latter possibility involved the study of serum influence on the effects produced on red blood cells. In these experiments some compounds belonging to non-steroidal anti-inflammatory drugs and deoxycholic acid were studied.

MATERIALS AND METHODS

The experiments were performed on New Zealand rabbits of both sexes weighing 2–3 kg.

Protection and lysis of erythrocytes in hypotonic solutions were determined by the rate of hemoglobin release, according to the procedure of Inglot and Wolna.[1] Red blood cell hemolysis in the presence of serum was studied by testing erythrocytes against rabbit serum diluted with sodium phosphate buffer (0·01 M; pH 7) in order to achieve a 50% hemolysis.

Drug protein binding was tested by the already described[4] ultrafiltration method using Sephadex G 25.

Moreover, benzydamine was determined by the method of Catanese *et al.*;[5] phenylbutazone by the method of Burns *et al.*;[6] indomethacin by the method of Hucker *et al.*,[7] and deoxycholic acid by the method of Levi *et al.*[8]

RESULTS

The effects of some drugs on erythrocyte hemolysis in buffer and serum are shown in Table 1. Results were reported by assuming a 100 value for controls presenting an average 50% hemolysis: values below 100 indicate, therefore, a decrease in hemolysis, while values above 100 are indicative of an increase in hemolysis.

TABLE 1. EFFECTS OF DRUGS ON ERYTHROCYTE MEMBRANE TESTED IN BUFFER SOLUTIONS AND IN THE PRESENCE OF SERUM

Drugs	Medium	Relative haemolysis (control = 100) at different mM concentrations		
		1	0·1	0·01
Benzydamine	buffer	158·7	62	92·1
	serum	193	66·1	92·6
Indomethacin	buffer	52·9	89·1	100
	serum	71·2	100	100
Deoxycholic acid	buffer	180	49·1	95·6
	serum	77·2	100	100
Phenylbutazone	buffer	79·9	100	100
	serum	82·7	100	100

As for the effects of drugs in buffer solutions, our results with indomethacin and phenylbutazone are in agreement with the data previously reported.[1] Deoxycholic acid and benzydamine were also observed to possess a stabilizing activity at concentrations between 0·01 mM and 0·1 mM, producing however hemolysis at 1 mM concentrations. In the presence of serum, the following results were achieved: the stabilizing effect of indomethacin was reduced; deoxycholic acid lost its hemolytic activity and stabilized the erythrocytes at higher concentrations than it did in buffer. As for benzydamine and phenylbutazone, no significant difference was observed in the results achieved with buffer compared with those achieved with serum.

Protein binding of drugs was studied during subsequent experiments, leading to the following results: benzydamine, less than 0·01mM; indomethacin, 0·07 mM; deoxycholic acid, 1·23 mM; phenylbutazone, 0·26 mM.

These results suggested that protein binding represented the limiting factor responsible for the drop of activity observed with some drugs in the presence of serum. Therefore, concentrations of drugs used to test their stabilizing effects on erythrocytes in the presence of serum have been processed in terms of free drug concentrations. An average 50% dilution of serum has been considered. Results obtained have been collected in Table 2.

On the basis of these figures, it may be concluded that protein binding of benzydamine and phenylbutazone does not significantly influence the quantity of free drugs at the given concentrations. In agreement with that, the activity of benzydamine and phenylbutazone is the same in aqueous solutions and in the presence of serum. With indomethacin and deoxycholic acid, protein binding significantly reduces their concentration, expressed in terms of free drug. This observation may explain the drop of activity observed following addition of serum.

DISCUSSION

Our experiments confirm phenylbutazone and indomethacin's stabilizing activity on erythrocyte membrane in the presence of buffer and evidence that benzydamine and deoxycholic acid possess a stabilizing activity at low concentrations, while they prove hemolytic at the highest concentrations used. In the presence of serum, the effects of benzydamine and phenylbutazone practically remained unchanged, whereas indomethacin lost any activity and deoxycholic acid only produced an erythrocyte

TABLE 2. FREE DRUG, EXPRESSED IN mM CONCENTRATION, PRESENT IN EXPERIMENTS CONDUCTED WITH RABBIT SERUM

Drugs	Free drug at different mM concentrations		
	1	0·1	0·01
Benzydamine	1	0·1	0·01
Indomethacin	0·29	0	0
Deoxycholic acid	0·36	0	0
Phenylbutazone	0·87	0	0

stabilization at the highest concentrations utilized. The protein-binding capacity of such drugs apparently explains the different results achieved with buffer as opposed to serum. In effect, benzydamine, which possesses a very low protein-binding capacity, has the same stabilizing effects on erythrocytes at the presence of both buffer or serum. The protein binding of phenylbutazone is of the order of 0·26 mM, but this value is low if compared with concentrations having a stabilizing effect on erythrocytes. Again, the effects on erythrocytes are basically the same both in buffer and serum (as a matter of fact, only 13 per cent of the drug is bound at the highest concentrations stabilizing erythrocytes in serum).

The situation is quite different with indomethacin. The lower concentration producing a stabilizing activity in buffer (0·1 mM) corresponds to a complete protein binding in serum: as a consequence the stabilizing activity is lacking in the presence of serum. The highest concentration investigated (1 mM) provides for indomethacin a protein binding of about 70 per cent, resulting consequently in a significant decrease in the stabilizing activity when passing from buffer to serum.

For deoxycholic acid a 0·1 mM concentration corresponds to a complete protein binding and the stabilizing effect detected in the presence of buffer is therefore lost in the presence of serum. At the concentration of 1 mM, about 64 per cent of the drug is bound to proteins: the hemolytic activity observed in buffer shifts then into a stabilizing activity in the presence of serum.

On the basis of these results, indomethacin and phenylbutazone can be considered as not-producing in humans any significant effect on red blood cells, since their blood levels at therapeutic doses are of 6 μg[7] and 100–150 μg/ml[6] of serum respectively: these amounts are very likely to be completely bound to serum proteins. As for benzydamine, man blood levels at therapeutic doses are of the order of 1 μg/ml,[5] such concentration being very close to that giving rise to a protective effect on the erythrocyte membrane. This drug, at least when completely free in human serum,[5] may be considered, therefore, as potentially endowed with a stabilizing effect on red

blood cells at therapeutic dosages. Deoxycholic acid requires a separate comment. Blood levels of bile salts are known to reach very high concentrations (300–350 μg/ml[9]) in the course of obstructive jaundice. According to the results obtained in buffer, deoxycholic acid should produce a hemolytic effect. The data achieved by studying the protein binding of such substance suggested, however, that deoxycholic acid is very likely to be bound to some extent to human protein serum: the free drug should produce, therefore, not a hemolytic but a stabilizing effect. According to clinical experiences, no hemolytic effect was observed in the course of obstructive jaundice, but rather an increase of red blood cell resistance to hemolysis.

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