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EFFECTS OF SOME *S*-ALKYLTHIOURONIUMS AND RELATED COMPOUNDS ON THE OSMOTIC FRAGILITY AND THE MEMBRANE EXPANSION OF HUMAN ERYTHROCYTES

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1 Changes in the osmotic fragility and critical haemolytic volume of human erythrocytes produced by *S*-*n*-decylthiouronium (S-10) and related compounds have been studied.

2 S-10 had a biphasic action on osmotic fragility, protecting erythrocytes against lysis in low concentrations but producing lysis in a concentration of 1 mM or higher.

3 Some lower homologues of S-10 also protected erythrocytes against osmotic lysis, the degree of protection depending on the length of the alkyl chain.

4 Critical haemolytic volume was increased by anthihaemolytic concentrations of procaine and chlorpromazine but not by antihaemolytic concentrations of S-10 and related amidines.

5 It is concluded that S-10 and its near homologues penetrate and stabilize erythrocyte membranes, potency increasing with the number of methylene groups in the side-chain up to about ten. The stabilization produced by S-10 apparently differs from that produced by many other lipid-soluble depressant drugs. It may be related to a drug-induced change in the ionic permeability of the membrane.

Introduction

Various pharmacological studies have been carried out with homologous series of amidines, especially the alkylguanidines and *S*-alkylthiouroniums (Fastier, 1962; Ozawa & Sugawara, 1968). Ascent of a series results in the intensification of actions which can be loosely described as 'depressant', e.g., vasodilator and other spasmolytic actions, inhibition of such enzymes as monoamine oxidase. This has been explained (Fastier & Reid, 1952) by supposing that some actions of amidines are not exerted in the same phase as the one in which the drug is administered. With increasing chain-length there should be an increasingly favourable distribution of the drug between an aqueous phase and the 'biophase' up to a cut-off imposed by insufficient solubility in water. It was subsequently suggested (Fastier, 1975) that depressant effects of amidinium ions are brought about through the hydrophobic portion of the molecule entering cell membranes and expanding them after the manner postulated by Seeman (1972) for various other lipid-soluble depressant drugs.

Some *S*-alkylthiouroniums and closely related compounds have therefore been tested for effects which they might be expected to produce if their depressant actions were due to stabilization of the cell membrane. Since the degree to which a compound can protect red cells against haemolysis is now commonly regarded as a measure of its membrane stabilizing ability, osmotic fragility experiments were performed with erythrocytes. The degree of membrane expansion was estimated by performing critical haemolytic volume experiments.

A preliminary account of this work was presented at a meeting of the Australian Physiological and Pharmacological Society (Beresford, 1976).

Methods

Osmotic fragility of erythrocytes

The osmotic fragility of red blood cells was estimated by measuring their resistance to lysis in saline solutions of decreasing concentration. The method adopted was a variation of that in current use in the Haematology Department of Dunedin Public Hospital and is based on the work of Parpart, Lorenz, Parpart, Gregg & Chase (1947). Buffered saline was used to maintain the pH at 7.4; the time of haemolysis was also controlled. All experiments were carried out at room temperature. The appropriate concentration of the drug being tested was added to each saline dilution followed by an aliquot of the blood sample. All samples were centrifuged 30 min later at

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300 *g* for 15 min. The clear supernatants were removed and the haemoglobin concentrations read at 540 λ on a Beckman spectrophotometer. The supernatant from the most concentrated saline tube was used as the internal standard. The haemoglobin content of the least concentrated saline solution was assigned the value of 100%; the concentrations of haemoglobin in all the other tubes were then expressed as a percentage of this value.

Critical haemolytic volume

A modification of the technique of Seeman, Sauks, Argent & Kwant (1969) was used to determine critical haemolytic volume (i.e., the packed cell volume immediately before haemolysis). Blood was withdrawn by venipuncture, heparinised and then centrifuged at 300 *g* for 15 min. Plasma and the buffy coat were removed. Next, the original blood volume was reconstituted with either saline buffer (0.85%) or drug dissolved in saline buffer. After they had been left for 30 min at room temperature, the tubes were again centrifuged for 15 min at 300 *g*, the supernatants being then discarded. Aliquots of red blood cells were pipetted into tubes containing either saline buffer alone (controls) or the drug dissolved in saline buffer. This buffer was prepared as for the osmotic fragility experiments and was used over the same range of concentrations. After thorough mixing of blood and buffer, microhaematocrit tubes were filled with the red cell mixture. All capillary tubes were sealed and spun at 15,000 *g* for 5 min in a microhaematocrit centrifuge. The packed cell volume of each tube was then read by means of a microhaematocrit reader. All haematocrit values were expressed as relative cell volumes, i.e., the value compared with that in 0.85% saline.

Materials used

Blood was obtained by venipuncture from three healthy human volunteers. Fresh blood was used for each experiment. The buffered saline had the following composition (mM): NaCl 1,500, Na_2HPO_4 100 and $NaHPO_4$ 15.6. It was diluted immediately before use to give final saline concentrations of 0.85% to 0.1% w/v. The following drugs were obtained from commercial sources: chlorpromazine hydrochloride (M&B); *n*-decylamine (I.C.N.); phenformin (Warner-Lambert); procaine hydrochloride (I.C.I.); *S*-*n*-decylthiouronium sulphate (I.C.N.). The following drugs were synthesized in our laboratory by reacting the corresponding alkyl bromide with thiourea in ethanol: *S*-*n*-hexyl, *S*-*n*-heptyl, *S*-*n*-octyl and *S*-*n*-nonyl-thiouronium bromides.

Statistical methods

Student's *t* test was used to assess the probability of differences between mean errors arising by chance. The measure of variability used was the standard error.

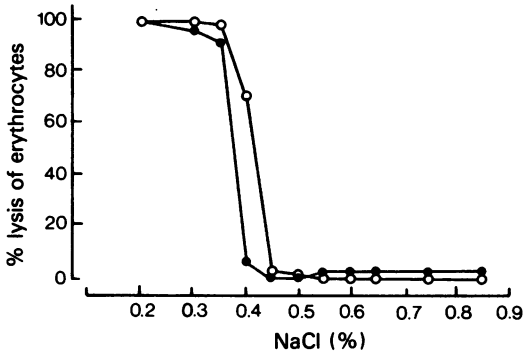

Figure 1 Reduction in the osmotic fragility of erythrocytes produced by *S*-*n*-decyl-thiouronium (40 μM): (○) cells in buffered saline; (●) cells in buffered saline containing 40 μM *S*-*n*-decyl-thiouronium. Results are all expressed as mean of 10 separate experiments; s.e. mean are all smaller than symbol size.

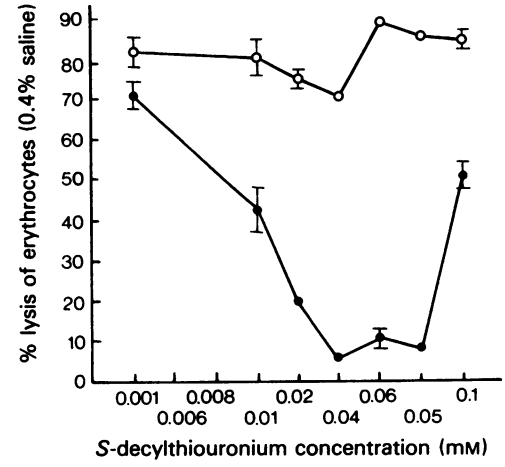

Figure 2 Effect of different concentrations of *S*-*n*-decyl-thiouronium on the percentage lysis (osmotic fragility) of erythrocytes: (○) cells in 0.4% saline, (●) cells in 0.4% saline containing *S*-*n*-decyl-thiouronium. Each point represents the mean of 10 experiments; vertical lines show s.e. mean where these are greater than the symbol size.

Results

S-*n*-decylthiouronium (S-10) had a biphasic effect on the osmotic fragility of erythrocytes, reducing it in low concentrations but increasing it in higher concentrations (Figures 1 and 2). Complete lysis was pro-

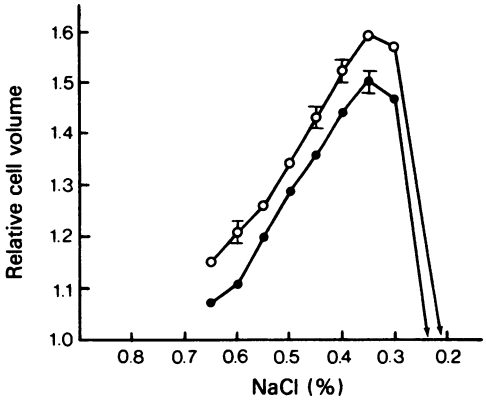

Figure 3 Effect of *S-n*-decyl-thiouronium (80 μM) on the relative cell volume or erythrocytes: (○) cells in normal saline, (●) cells in saline containing *S-n*-decyl-thiouronium. Each point represents the mean of 10 experiments, vertical lines show s.e. mean. The maximum volume attained by the packed cells before lysis occurs is the critical haemolytic volume of those erythrocytes. Comparison with the result obtained in 0.85% saline gives the relative cell volume.

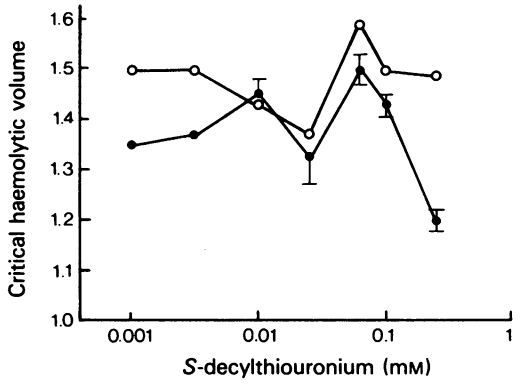

Figure 4 Effect of a range of antihaemolytic concentrations of *S-n*-decyl-thiouronium on the critical haemolytic volume of erythrocytes: (○) cells bathed in normal saline; (●) cells bathed in saline containing *S-n*-decyl-thiouronium. Results are expressed as mean of 10 experiments; vertical lines show s.e. mean.

duced by concentrations of 1 mM and higher. No concentration of the drug increased the critical haemolytic volume of the erythrocytes. In fact, some concentrations of S-10 decreased it significantly (Figures 3 and 4).

Since the effect of S-10 on critical haemolytic volume was not that predicted, the technique was checked by carrying out experiments with two drugs known to increase critical haemolytic volume, procaine (Roth & Seeman, 1972) and chlorpromazine (van Steveninck, Gjösund & Booij, 1966). Each was found to increase critical haemolytic volume when given in a concentration which reduced osmotic fragility (Table 1).

The primary amine corresponding to *S*-10, *n*-decylamine, lysed erythrocytes when given in a concentration of 1 mM but reduced fragility when given in a concentration of 0.1 mM. It did not affect critical haemolytic volume. Phenformin has no antihaemolytic effect, so it was not tested for ability to expand the erythrocyte membrane.

Four lower homologues of S-10 were tested, *S-n*-hexyl-, *S-n*-heptyl-, *S-n*-octyl- and *S-n*-nonyl-thiouronium (S-6, S-7, S-8 and S-9). S-6 did not produce any antihaemolysis. Higher homologues did reduce osmotic fragility, the minimum effective concentration declining from 1 mM for S-7 to 1μM for S-10 (Figure 5). Each homologue was tested at the concentration which produced the greatest antihaemolysis for its effect on critical haemolytic volume. S-7 and S-8 were without effect. The effect of S-9 varied; whereas a concentration of 0.007 mM increased critical haemolytic volume, one of 0.04 mM decreased it. Table 2 summarizes the effects of all amidines tested.

Discussion

Human red cells contain protective mechanisms for

Table 1 Effects of chlorpromazine and procaine on the critical haemolytic volume of erythrocytes

Compound	*Antihaemolytic concentration (mM)*	*Control*	*Test*
Chlorpromazine	0.1	1.34 (0.01)	1.45 (0.01)**
Chlorpromazine	0.01	1.34 (0.01)	1.45 (0.007)**
Procaine	0.01	1.40 (0.01)	1.45 (0.07)*

The results are expressed as the mean (s.e. mean) for 8 to 10 experiments.
Significance of results, as determined by Student's *t* test, denoted by * for $P < 0.05$ and by ** for $P < 0.001$.

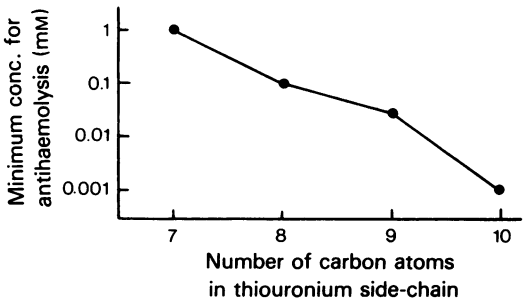

Figure 5 Reduction in the minimum concentration of drug required to produce antihaemolysis as a consequence of increasing the number of methylene groups in the thiouronium side chain. C_7 is *S-n*-heptyl-thiouronium, C_8 is *S-n*-octyl-thiouronium, C_9 is *S-n*-nonyl-thiouronium and C_{10} is *S-n*-decyl-thiouronium.

the maintenance of membrane integrity. Although they have a normal life span of about 120 days, they can be lysed at any stage by mechanical or osmotic means. A variety of drugs, most of them lipid-soluble, can protect erythrocytes against osmotic lysis. Amongst known protective agents are amyl alcohol (Traube, 1908), several volatile anaesthetics (Jacobs & Parpart, 1932), tranquillizers and antihistaminics (Seeman & Weinstein, 1966), steroids and anti-inflammatory drugs (Seeman, 1966a, Inglot & Wolna, 1968; Brown, Taylor & Waters, 1971) fat-soluble vitamins (Seeman, 1966b) and propranolol (Fortier, Snyder, Palek & Weiss, 1977). Seeman (1972) has shown that the concentrations of anaesthetics which produce 50% antihaemolysis are those blocking conduction in nerves.

All these drugs have a biphasic action, being protective at low concentrations (0.01 μM to about 1 mM) and lytic at higher concentrations. A few which appear to produce stabilisation only, e.g., Δ-9-tetrahydrocannabinol (Chari-Bitron, 1971), have usually been tested only in fairly low concentrations. The lipid-soluble stabilizers differ from specific lysins, such as saponin and filipin, which at all concentrations disrupt erythrocyte membranes by dissolving out cholesterol (Seeman, 1974).

Table 2 Effects of *S-n*-decylthiouronium and some related compounds on erythrocyte membranes

Compound	*Concentration (mM)*	*Effect on osmotic fragility*	*Effect on critical haemolytic volume**
S-n-decyl-thiouronium	1	Lysis	
	0.1	Antihaemolysis	Significant decrease
	0.04	Antihaemolysis	NS
	0.01	Antihaemolysis	NS
	0.004	Antihaemolysis	Significant decrease
	0.001	NS	
S-n-nonyl-thiouronium	1	Lysis	
	0.07	Antihaemolysis	Significant increase
	0.04	Antihaemolysis	Significant decrease
	0.01	NS	
S-n-octyl-thiouronium	1	Lysis	
	0.1	Antihaemolysis	NS
	0.1	NS	
S-n-heptyl-thiouronium	5	Lysis	
	1	Antihaemolysis	NS
	0.1	NS	
S-n-hexyl-thiouronium	0.1	Lysis	
	0.01	NS	
n-Decylamine	1	Lysis	
	0.1	Antihaemolysis	NS
Phenformin	1	NS	
	0.1	NS	
	0.01	NS	

* Critical haemolytic volume studies were carried out only with those concentrations of a drug which had been shown to be antihaemolytic. NS = not significant.

Our experiments have shown that S-10, too, has a biphasic action on erythrocyte membranes. As anticipated, low concentrations of the drug were found to stabilize the cell membrane. The increase in stabilizing activity from the *S-n*-hexyl to the *S-n*-decyl derivative was also expected. The importance of a long methylene chain is borne out by the activity of *n*-decylamine and the inactivity of phenformin (2-phenylethyldiguanide).

Several explanations for the antihaemolytic activity of so many different drugs have been advanced. They include a reduction in the passive inflow of water (Freeman & Spirtes, 1962), an increase in the outflow of potassium ions (Passow & Tillman, 1956), and a reduction in the inflow of sodium ions (van Steveninck *et al.*, 1966). None of these hypotheses was supported by the results of van Steveninck *et al.* (1966). However, when Ponder's hypothesis that 'sublytic concentrations of anaesthetics might increase critical haemolytic volume' (Ponder, 1948) was tested by van Steveninck and his associates, they found that chlorpromazine increased the critical haemolytic volume of erythrocytes by about 10%. Seeman, Kwant, Sauks & Argent (1969) found a similar, though smaller, increase in critical haemolytic volume with chlorpromazine and all other tranquillizing and local anaesthetic agents tested.

Measurement of changes in membrane density (Seeman, 1974; 1975) have shown that local and general anaesthetics increase the volume of cell membranes as well as their area. Since the membrane expansion observed is about twenty times the occupying volume of any drug contained in the membrane phase, the drug molecules alone could not account for this increase in volume. Seeman (1972) has therefore suggested that the observed increase in membrane area and volume might be due to expansion of membrane proteins. In his view, adsorption of local anaesthetics and other drugs on hydrophobic regions of excitable membranes would expand the membrane proteins and thus block ionic conductance channels. Other workers have confirmed that anaesthetics do not affect the ordering of lipid bilayers (Boggs, Yoong & Hsia, 1976) but instead act primarily on membrane proteins, both polar and non-polar regions of the proteins being involved as binding sites (Franks & Lieb, 1978). However, Seeman's membrane-expansion theory of anaesthesia has not won complete acceptance. As Paton (1975) has remarked, membrane expansion may occur during anaesthesia but hardly provides a mechanism for the phenomenon.

Our results show that antihaemolysis is not necessarily associated with membrane expansion, at any rate as indicated by a increase in critical haemolytic volume. In fact a 6% reduction was obtained in experiments with S-10. This would correspond to a reduction of about 3% in membrane area, but we were unable to measure changes in membrane area directly for lack of a high-speed density meter. Likewise with lower homologues of S-10, no increase in critical haemolytic volume was obtained when antihaemolytic concentrations were used except at one concentration of S-9. That the technique was not at fault was confirmed by the results obtained with procaine and chlorpromazine. Some factor other than membrane expansion must therefore be involved in the stabilizing action of S-alkylthiouroniums on the erythrocyte membrane.

We are not alone in demonstrating that antihaemolysis can occur in the absence of membrane expansion. Fortier *et al.* (1977) showed that antihaemolytic concentrations of propranolol could reduce mean corpuscular volume by almost 20%. They found that potassium was lost from the cells during this reduction in volume and that the fragility changes could be prevented by the addition of potassium. Hence their observations were in accord with one of the hypotheses that had apparently been refuted by the work of van Steveninck *et al.* (1966) and by Roth & Seeman (1972).

A similar efflux of potassium might well occur when erythrocytes are treated with S-10. Hughes & Macknight (1975), working with slices of rat renal cortex, observed that cells treated with S-10 lost significantly more potassium than did control cells during leaching at low temperature. The loss was reversible in cells treated with a low concentration (0.1 mM) but irreversible when a higher concentration (1 mM) was used. The higher concentration is lytic to erythrocytes, as we have just shown. It has also been found in experiments on skeletal muscle to uncouple oxidative phosphorylation, possibly by acting on the mitochondrial membrane (Beresford, Bills, Fastier & Milne, 1979).

The stabilizing effect of lower concentrations of S-10 might well be associated with an ability to penetrate cell membranes. In so doing S-10 may bring about a leakage of potassium from the cell, this being responsible for the observed reduction in critical haemolytic volume. However, an effect on sodium permeability cannot be ruled out, because some unspecific membrane depressants are thought to reduce membrane permeability to sodium as a result of an ability to displace calcium from binding sites (Feinstein & Paimre, 1969; Seeman, 1972). S-10 and calcium have been shown to have antagonistic actions on smooth muscle (Bills & Fastier, 1973). Inhibition of cation-dependent membrane ATPases by S-10 and other *S*-alkyl-thiouroniums has also been demonstrated (Thomas, Gelbart, Grigor, Harvey & Fastier, 1973). The reduction in critical haemolytic volume obtained with antihaemolytic concentrations of S-10 may therefore be due not only to an increased efflux of potassium ions from the cell but also to a de-

creased influx of sodium ions. Such an unspecific action on membrane permeability would explain why S-10 has been found in experiments on smooth muscle preparations (Fastier & Reid, 1952; Corbett, Bills & Fastier, 1974) to antagonize almost equally stimulant actions of acetylcholine, histamine, adrenaline and calcium.

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