# Stabilization of erythrocyte membrane by non-steroid anti-inflammatory drugs

An intensive anti-denaturing (stabilizing) effect of anti-inflammatory drugs at therapeutic concentrations on serum proteins has previously been reported by Mizushima & Kobayashi (1968) and Mizushima (1968). Silvestrini & Catanese (1968) obtained a similar effect. Brown, Mackey & Riggilo (1967) and Inglot & Wolna (1968) reported that non-steroid anti-inflammatory drugs such as phenylbutazone and indomethacin stabilized canine and human erythrocytes to heat-induced and hypotonic haemolysis. These findings suggest that stabilization of the erythrocyte membrane by anti-inflammatory drugs may arise from a stabilizing effect of the drugs on some proteins in the cell membrane.

Skidmore & Whitehouse (1965) found that the heat denaturation (coagulation) of serum albumin treated with 2,4,6-trinitrobenzene sulphonic acid (TNBS) was no longer inhibited by anti-inflammatory drugs. Since TNBS reacts with the lysyl $\epsilon$-amino-group of albumin, Skidmore & Whitehouse believed that anti-inflammatory drugs became bound to the $\epsilon$-amino-group and thus stabilized albumin. We have now found canine erythrocytes treated with TNBS to be no longer stabilized to heat-induced haemolysis by anti-inflammatory drugs like phenylbutazone.

After the method of Brown & others (1967), blood was removed from mongrel dogs by a heparinized syringe. The plasma was discarded and the erythrocytes washed twice with saline. Four ml of 40 mM TNBS in saline was added to 36 ml of 5% suspension of the erythrocytes in 0·2 M bicarbonate buffer at pH 8·1. After incubation for 90 min at room temperature (20°), the suspension was centrifuged. The treated erythrocytes were washed with saline and resuspended in 36 ml of 0·15 M phosphate buffer at pH 7·4. To 2·7 ml of the TNBS-treated erythrocyte suspension, or non-treated 5% erythrocyte suspension in the phosphate buffer, were added 0·3 ml of 5 mM anti-inflammatory drugs in saline (pH 7), or saline alone. After standing at room temperature for 15 min, the mixtures were heated in a water bath at 50–51° for 20 min. Samples were cooled with water and promptly centrifuged for 20 min at 14,000 *g*. The haemoglobin content of clear supernatants was measured with a spectrophotometer at 540 nm. The heated control tubes both of non-treated and TNBS groups showed a reading of the spectrophotometer at –log transmission 0·7–1·0 in the method used. Mean haemoglobin content of the heated control tubes, from which the haemoglobin content of the corresponding unheated control tubes had been subtracted, was expressed as 100, as a relative amount of haemolysis. The relative amount of haemolysis of each tube tested was calculated. Erythrocytes treated with 4 mM TNBS were no longer or only minimally stabilized by anti-inflammatory drugs against heat-induced haemolysis, while non-treated erythrocytes were strongly stabilized by them (Table 1).

Table 1. *Effect of TNBS on stabilization of erythrocytes by anti-inflammatory drugs against heat-induced haemolysis*

| | Relative amount of haemolysis and standard error | |
|---|---|---|
| | non-treated erthrocytes | TNBS-treated erythrocytes |
| None (control) .. .. .. .. | 100 ± 3·2 (7*, 19**) | 100 ± 1·5 (7, 24) |
| Phenylbutazone .. .. .. .. | 25 ± 2·8 (7, 16) | 91 ± 3·3 (7, 18) |
| Flufenamic acid .. .. .. .. | 34 ± 3·1 (5, 10) | 109 ± 4·0 (3, 11) |
| Indomethacin .. .. .. .. | 7 ± 3·2 (4, 9) | 103 ± 4·4 (4, 11) |

4·5% of canine erythrocytes treated with and without 4 mM 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) were heated for 20 min at 50–51°C with and without 0·5 mM anti-inflammatory drugs.
* No. of animals used.
** No. of samples tested.

It seems, therefore, that stabilization of the erythrocyte membrane by anti-inflammatory drugs is probably due to a stabilizing effect of the drugs on some proteins in the cell membrane in a manner similar to that observed by us in the experiments on protein denaturation (Mizushima, 1968).

We are grateful to Kowa Ltd., Nagoya for financial support.

*Department of Physical Therapy and Medicine,*
*University of Tokyo,*
*Tokyo, Japan.*
Y. MIZUSHIMA
S. SAKAI

January 25, 1969

REFERENCES

BROWN, J. H., MACKEY, H. K. & RIGGILO, D. A. (1967). *Proc. Soc. exp. Biol. Med.*, **125**, 837–842.
INGLOT, A. D. & WOLNA, E. (1968). *Biochem. Pharmac.*, **17**, 269–279.
MIZUSHIMA, Y. (1968). In *Inflammation*, p. 37–44, ICS 163, Amsterdam: Excerpta Medica.
MIZUSHIMA, Y. & KOBAYASHI, M. (1968). *J. Pharm. Pharmac.*, **20**, 169–173.
SILVESTRINI, B. & CATANESE, B. (1968). *Arzneimittel-Forsch.*, **18**, 425–426.
SKIDMORE, I. F. & WHITEHOUSE, M. W. (1965). *J. Pharm. Pharmac.*, **17**, 671–673.

# Inhibition of the noradrenaline uptake in guinea-pig vas deferens by continuous nerve stimulation

The release and the uptake of noradrenaline by nerve terminals can be modulated by different regulatory mechanisms. Gillis (1963) observed that short-lasting stimulation of sympathetic nerves increased the uptake of noradrenaline by the cat perfused heart. Blakeley & Brown (1964) found that nerve stimulation produced an inhibition of the uptake of noradrenaline infused into the cat spleen. Chang & Chiueh (1968) demonstrated that intermittent nerve stimulation of the branches of the facial nerve increased the uptake of tritiated noradrenaline ($^3$H-NA) in rat submaxillary gland. We now report how continuous nerve stimulation with different frequencies can affect the noradrenaline uptake. The hypogastric nerve-vas deferens preparation was chosen because of the abundance of adrenergic synapses in this organ and the opportunity it offers for pre- and postganglionic stimulation (Birmingham & Wilson, 1963).

Male guinea-pigs of about 400 g provided the vasa deferentia and hypogastric nerve preparations which were incubated in a 50 ml bath in modified Krebs solution (Huković, 1961) at 37° for 30 min. The vas deferens from one side was stimulated while that on the other side served as control. The electrical stimulation of the hypogastic nerve was performed as described by Huković (1961), and transmural stimulation as described by Birmingham & Wilson (1963). Monophasic square stimuli of supramaximal strength and 1 ms duration were applied continuously for 30 min. The contractions of the vas deferens were recorded on smoked paper. For the transmural stimulation the vas deferens was dissected and cleaned of peritoneum and surrounding fat tissue.

$^3$H-NA (specific activity 190 mCi/mg) was added to the bath (final concentration, 5 ng/ml of medium). According to Avakian & Gillespie (1968) this concentration is low enough to ensure the uptake of noradrenaline exclusively by the nerve terminals. Oxidation of $^3$H-NA was prevented by the addition of EDTA (10 mg/litre) and ascorbic acid (20 mg/litre). The pH of the medium was 7·4. After incubation, the preparations were washed twice in 5 ml of saline for 30 s and blotted dry on filter paper. The tissue was homogenized in 0·4N perchloric acid. $^3$H-NA was separated on alumina columns (Whitby, Axelrod & Weil-Malherbe, 1961). The [$^3$H]radioactivity was measured by a Packard liquid scintillation counter. The amount of $^3$H-NA metabolites was calculated by subtracting the specific $^3$H-NA radioactivity from the total [$^3$H]radioactivity.