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## References

Burn, J. H. (1952). *Practical Pharmacology*, pp. 35–38, Oxford: Blackwell.
Zarro, V. J. & DiPalma, J. R. (1964). *J. Pharm. Pharmacol.*, **16**, 427–428.

## Concerning the regulation of some diverse biochemical reactions, underlying the inflammatory response, by salicylic acid, phenylbutazone and other acidic antirheumatic drugs

SIR,—Recent *in vitro* studies of the properties of non-steroid anti-inflammatory: antirheumatic drugs have indicated that, in addition to their analgesic properties, they might inhibit several chemical reactions *in vivo* which probably participate in the overall development of *signa inflammationis*, the subsequent formation of granulation (scar) tissue and wound repair (Spector, 1964; Garattini & Dukes, 1965; Whitehouse, 1965). The biochemical reactions include (i) the mitochondrial biosynthesis of adenosine-5′-triphosphate(ATP) ("oxidative phosphorylation"); (ii) the formation of histamine by substrate-specific histidine decarboxylase(s); (iii) the hydrolysis of proteins or amino-acid esters, or both, by enzymes resembling trypsin (EC no. 3.4.4.4) in their substrate-specificity, for example, the Hageman factor (Schoenmakers, Matze, Haaner & Zilliken, 1964), the kinin-forming enzymes (Webster & Pierce, 1961), and thrombin and plasmin: fibrinolysin (Scheraga, Ehrenpreis & Sullivan, 1958).

These three particular processes, though superficially unrelated in their chemistry, appear to have at least two features in common: firstly their sensitivity to mM concentrations of salicylic acid and certain other acidic anti-inflammatory drugs such as phenylbutazone, cinchophen, indomethacin and flufenamic acid; and secondly, the involvement of an $\epsilon$-amino-group (belonging to a lysine residue in the enzyme protein or protein substrate) in the enzyme-substrate interaction. Where the relation between chemical structure and the ability to inhibit these enzyme reactions has been investigated (see Whitehouse, 1965), it is notable that non-acidic derivatives of these drugs, such as salicylamide, *N*-arylanthranilamides and the amide of indomethacin, are unable to substitute for the parent acid as effective anti-inflammatory drugs or inhibitors of these enzyme systems (although they may still carry analgesic activity). We therefore believe that some of the apparently diverse effects of salts of salicylic acid and other anti-inflammatory acids upon enzyme systems, especially those implicated in the inflammatory response of animal tissues, are due to "neutralisation" of essential lysyl $\epsilon$-amino-groups by the anionic form of these drugs. The evidence for this hypothesis is summarised, as follows.

A. Drugs known to selectively inhibit mitochondrial ATP biosynthesis without affecting mitochondrial respiration (so-called "uncoupling agents") are either (i) acids able to partition from an aqueous phase into the mitochondrial lipid phase, for example, 2,4-dinitrophenol and the acidic anti-inflammatory drugs considered here, or (ii) compounds able to interact with an amino-group adjacent to a thiol group, for example, certain trivalent arsenicals, and carbonyl-cyanide-phenylhydrazones (Heytler, 1963; Whitehouse, 1965). We discovered that several other compounds able to react with free amino-groups under mild conditions (quasi-physiological pH, room temperature) in an aqueous medium,

selectively uncouple oxidative phosphorylation at concentrations which have little or no effect upon either electron transport (succinate oxidation) by rat liver mitochondria or on the activity of added yeast hexokinase (used to trap newly-synthesised ATP) under the experimental conditions described elsewhere (Skidmore & Whitehouse, 1965a). These uncoupling "amine reagents" include 2,4,6-trinitrobenzene sulphonic acid (4 mM), 1-fluoro-2,4-dinitrobenzene (FDNB, 0·8 mM), 2-nitrobenzaldehyde and cinnamaldehyde (5 mM), 2,4,6-trinitrobenzaldehyde (0·5 mM), and also ninhydrin (0·2 mM). Furthermore, the uncoupling effect of some of these reagents (e.g. 5 mM 2-nitrobenzaldehyde or FDNB, 2·5 mM trinitrobenzaldehyde) was not wholly reversed by tenfold dilution after preincubation with buffered mitochondria alone (7 min at 30°); by contrast, the uncoupling action of acidic drugs (80 $\mu$M 2,4-dinitrophenol, 3 mM salicylic acid, 0·5 mM indomethacin) and carbonylcyanide *m*-chlorophenylhydrazone (4 $\mu$M) was completely reversed under these conditions. This suggests that these "amine reagents" were not inhibiting phosphorylation merely as a consequence of oxidation or hydrolysis to carboxylic or phenolic acids within mitochondria. Preliminary chemical analyses support this conclusion. Other experiments indicated that the 6-amino-group of the adenine moiety (of ATP-precursors) was not blocked by these particular amine reagents under conditions that brought about uncoupling of oxidative phosphorylation.

It seems reasonable to infer that a lysyl $\epsilon$-amino group might be implicated in mitochondrial ATP biosynthesis. This process probably provides the bulk of the ATP utilised in various endergonic reactions associated with the inflammatory response, for example, in histamine release (Uvnäs, 1964), or in connective-tissue biosynthesis.

B. We have observed that salicylic acid, indomethacin, phenylbutazone and flufenamic acid (at $\leqslant$ 1 mM) all inhibit histamine formation *in vitro* by animal enzymes (from rat pyloric stomach, or foetal liver) which specifically decarboxylate L-histidine and also have a relatively low affinity for the coenzyme, pyridoxal phosphate ($K_m \geqslant 0{\cdot}1$ $\mu$M). At concentrations below 10 mM these drugs do not inhibit the non-specific animal L-aromatic amino-acid decarboxylase (for example, from guinea-pig kidney) or a bacterial histidine decarboxylase (from *Cl. welchii*) which have a higher affinity for pyridoxal phosphate. This drug inhibition of the animal substrate-specific enzymes is non-competitive with respect to the substrate and these drugs appear to act by releasing bound pyridoxal phosphate from the decarboxylase enzyme. Chemical analogues of these drugs which are devoid of anti-inflammatory activity (such as benzoic acid, phenazone, 5-methoxyindole-3-acetic acid) have little or no effect upon histamine formation *in vitro*.

Transaminases, which contain pyridoxal phosphate linked to a lysyl $\epsilon$-amino-group by an azomethine linkage (Braunstein, 1964), are also inhibited by salicylic acid, cinchophen and phenylbutazone (Pulver, Exer & Herrmann, 1956; Huggins, Smith & Moses, 1961; Hänninen & Hartiala, 1965). Pyridoxal phosphate binds to plasma albumen through a similar linkage (Dempsey & Christensen, 1962) and may be displaced from the albumen amino-groups by these acidic anti-inflammatory drugs (Skidmore & Whitehouse, 1965b).

C. Trypsin and non-enteric enzymes with a similar substrate specificity (for example, cathepsin B, Hageman factor, plasmin) may provoke inflammation (Domenjoz & Mörsdorf, 1965; Graham, Ebert, Ratnoff & Moses, 1965). They all hydrolyse peptide or ester linkages in which an L-arginine or L-lysine residue contributes the carbonyl group (Neurath & Schwert, 1950; Scheraga & others, 1958; Schoenmakers & others, 1964). Tryptic digestion *in vitro* of certain proteins, (like diazotised collagen or rat paw homogenates, is partially

inhibited by salicylic acid, phenylbutazone, indomethacin and flufenamic acid and also by amidopyrine (Bertelli, Donati & Rossano, 1965; Mörsdorf, 1965). Domenjoz and Mörsdorf (1965) reported that these acidic drugs also inhibit trypsin-induced oedema in rats.

We found that anti-inflammatory acids lower the initial rate of tryptic digestion of ethanol-denatured horse heart cytochrome *c* (Sigma Chemical Co., London) and urea-denatured globin (Edmundson & Hirs, 1962) prepared from horse heart myoglobin (British Drug Houses Ltd., Poole = B.D.H.). The ratio of lysine residues to arginine residues is 8·5 in purified myoglobin (Holleman and Biserte, 1959) and 9·5 in the cytochrome *c* (Margoliash, Kimmel, Hill and Schmidt, 1962). Tryptic digestion of a commercial protamine preparation (salmine sulphate, B.D.H.), containing 53% arginine and only 0·2% lysine, was far less sensitive to these drugs. With 0·14 mM globin (2·5 mg/ml), equivalent to 2·4 mM lysyl $\epsilon$-amino-groups, and 10 $\mu$g/ml crystalline trypsin (B.D.H.) in 60 mM Tris hydrochloride, pH 7·4 or 7·9, the initial rate of proteolysis (up to 4 min) was $\leqslant$ 50% of that in drug-free controls in the presence of 15 mM sodium salicylate or 3 mM phenylbutazone or 2 mM indomethacin or 4 mM 2,4,6-trinitrobenzaldehyde (all added in pre-neutralised solution at pH 7·4 or 7·9). With 1·3 mg/ml salmine sulphate and 5 $\mu$g/ml trypsin, the same concentrations of these drugs lowered the initial rate of tryptic protamine digestion by not more than 20% at pH 7·9 and even less at pH 7·4. Three basic anti-inflammatory drugs, chloroquine (20 mM), amidopyrine (20 mM) and hexadimethrine hydrobromide (Polybrene, 2 mg/ml), had no effect on tryptic digestion of protamine and globin.

Presumably concentrations of the acidic drugs lower than those given above would appreciably inhibit non-enteric tryptic enzymes which hydrolysed lysyl (rather than arginyl) linkages in proteins, if either their protein substrates were available only at concentrations giving an effective concentration of lysyl $\epsilon$-amino-groups less than this figure (2·4 mM) or these non-enteric enzymes had lower affinities for their substrates than trypsin has for (myo)globin, or both. Rather high $K_m$ values (70, 17 mM) have been reported for the hydrolysis of lysine ethyl or methyl esters by thrombin and plasmin respectively (Scheraga & others, 1958). We determined the $K_m$ for tryptic hydrolysis of lysine methyl ester to be 0·2 mM at pH 7·4 or 7·9 and 20°, which suggests that trypsin may have a higher affinity for lysyl protein substrates than do non-enteric enzymes such as thrombin or plasmin. If so, these latter enzymes would compete less successfully with an acidic drug for the lysyl (amino-group) binding sites than does trypsin; the drugs should then be more effective in controlling hydrolysis of peptide linkages adjacent to lysine residues by non-enteric (pseudo) tryptic enzymes than they are in inhibiting protein digestion by trypsin itself.

To summarise our hypothesis: the lysyl $\epsilon$-amino-groups of certain proteins appear to be important binding sites for acidic anti-inflammatory drugs, where they may then interfere with enzymic reactions dependent upon the availability of these $\epsilon$-amino-groups, either for pyridoxal phosphate binding (for example, histamine formation), or directing the enzyme reaction (for example, tryptic-like proteolysis, or mitochondrial phosphorylation).

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## References

Bertelli, A., Donati, L. & Rossano, M. A. (1965). In *Non-steroidal anti-inflammatory drugs*, Editors Garattini, S. & Dukes, M. N. G., pp. 98–102.
Braunstein, A. E. (1964). *Vitam. & Horm.*, **22**, 452–457.
Dempsey, W. B. & Christensen, H. N. (1962). *J. biol. Chem.*, **237**, 1113–1120.
Domenjoz, R. & Mörsdorf, K. (1965). In *Non-steroidal anti-inflammatory drugs*, Editors Garattini, S. & Dukes, M. N. G., pp. 162–173.
Edmundson, A. B. & Hirs, C. H. W. (1962). *J. mol. Biol.*, **5**, 666.
Garattini, S. & Dukes, M. N. G. (1965). *Non-steroidal anti-inflammatory drugs*. Amsterdam: Excerpta Medical Foundation.
Graham, R. C. Jr., Ebert, R. H., Ratnoff, O. D. & Moses, J. M. (1965). *J. exp. Med.*, **121**, 807–818.
Hänninen, O. & Hartiala, K. (1965). *Biochem. Pharmacol.*, **14**, 1073–1076.
Heytler, P. G. (1963). *Biochemistry*, **2**, 357–361.
Holleman, J. W. & Biserte, G. (1959). *Biochem. Biophys. Acta*, **33**, 143–149.
Huggins, A. K., Smith, M. J. H. & Moses, V. (1961). *Biochem. J.*, **79**, 271–275.
Margoliash, E., Kimmel, J. R., Hill, R. L. & Schmidt, W. R. (1962). *J. biol. Chem.*, **237**, 2148–2150.
Mörsdorf, K. (1965). In *Non-steroidal anti-inflammatory drugs*, Editors Garattini, S. & Dukes, M. N. G., pp. 85–89.
Neurath, H. & Schwert, G. W. (1950). *Chem. Revs.*, **46**, 104–110.
Pulver, R., Exer, B. & Herrmann, B. (1956). *Schweiz. med. Wschr.*, **86**, 1080–1085.
Scheraga, H. A., Ehrenpreis, S. & Sullivan, E. (1958). *Nature*, **182**, 461–462.
Schoenmakers, J., Matze, R., Haanen, C. & Zilliken, F. (1964). *Biochim. Biophys. Acta*, **93**, 433–436.
Skidmore, I. F. & Whitehouse, M. W. (1965a). *Biochem. Pharmacol.*, **14**, 547–555.
Skidmore, I. F. & Whitehouse, M. W. (1965b). *J. Pharm. Pharmacol.*, **17**, 671–673.
Spector, W. G. (1964). Consulting Editor *Ann. N.Y. Acad. Sci.*, **116**, 747–1084.
Uvnäs, B. (1964. In Spector, W. G. (1964), pp. 880–890.
Webster, M. E. & Pierce, J. V. (1961). *Proc. Soc. exp. Biol. N.Y.*, **107**, 186–191.
Whitehouse, M. W. (1965). In *Progr. Drug. Res.*, **8**, 323–429.

## Effect of non-steroid anti-inflammatory drugs on aldehyde binding to plasma albumen: a novel *in vitro* assay for potential anti-inflammatory activity

SIR,—Dempsey & Christensen (1962) showed that pyridoxal phosphate binds to the lysyl $\epsilon$-amino-groups of plasma albumen and that certain aromatic acids, including salicylic acid, partly reversed this association. We have confirmed and extended their observations as follows. When bovine plasma albumen (Armour Pharmaceuticals Ltd., Eastbourne) was treated with 2,4,6-trinitrobenzene sulphonic (picryl sulphonic) acid (TNBS) in aqueous solution at pH 7·5, at least 50 amino-groups per albumen molecule (number average) were substituted by the trinitrophenyl (TNP) group. The product, "TNP-albumen", no longer bound salicylate anions or pyridoxal phosphate. A similar loss of binding capacity occurred when albumen was treated with 1-fluoro-2,4-dinitrobenzene (FDNB, Sanger's reagent) in dilute sodium bicarbonate solution at pH 8·5. Albumen which had been dialysed against a solution of sodium salicylate at pH 7·5 and had bound *several* molecules of salicylate per albumen molecule, lost all the bound salicylate on treatment with TNBS or FDNB. These findings establish the lysyl $\epsilon$-amino-group as a common binding site for both the salicylate ion and pyridoxal phosphate. The single *N*-terminal aspartyl $\alpha$-amino-group which also reacts with TNBS and FDNB, could only bind one molecule of drug or coenzyme. [TNBS and FDNB do not react with the guanidino-group of arginine, the only other basic residue in the albumen molecule].

Many acidic anti-inflammatory drugs inhibit mammalian substrate-specific histidine decarboxylases *in vitro* (Whitehouse & Skidmore, 1965); this is believed