AB HÄSSLE, Göteborg and
Department of Pharmacology,
University of Göteborg.

H. CORRODI

Department of Histology,
Karolinska Institutet,
Stockholm, Sweden.

K. FUXE
T. HÖKFELT

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

SIR,—Under this title, we proposed the hypothesis that lysyl ϵ-amino-groups of certain proteins are important binding sites for acidic anti-inflammatory drugs, and that when so bound these drugs may inhibit enzymic reactions which depend upon the availability of these ϵ-amino-groups—either for binding an essential cofactor (for example pyridoxal phosphate) or for directing the enzymic reaction (for example tryptic-like proteolysis or mitochondrial phosphorylation) (Whitehouse & Skidmore, 1965). We now have evidence that these particular drugs may also influence other biochemical reactions which might be involved in the inflammatory response, by acting as pseudo-antimetabolites and inhibiting the metabolism of certain derivatives of aromatic amino-acids.

Enzymes resembling chymotrypsin (E.C. no. 3.4.4.5) in their substrate specificity have been found in the granules of rat mast cells (Lagunoff & Benditt, 1963) and have been implicated in the swelling of the paw of the rat after the local injection of an inflammatory agent (Hladovec & Rybák, 1963) and also in the anaphylactic release of histamine either from sensitised lung of the guinea-pig (Austen & Brockelhurst, 1961a) or from rat peritoneal mast cells (Keller, 1963). Chymotrypsin hydrolyses esters and amides of aromatic amino-acids, leucine, methionine and even histidine (Kloss & Schröder, 1964) as well as proteins, and releases histamine from mast cells with concomitant degranulation (Üvnas & Antonsson, 1963; Saeki, 1964). Sodium salicylate inhibits a guinea-pig lung protease (Ungar, Yamura, Isola & Kobrin, 1961) and the anaphylactic release of histamine in guinea-pigs (Mongar & Schild, 1957) and rabbits (Haining, 1956). Mörsdorf, Donner & Cornellisson (1966) found that the *N*-acetyltyrosine esterase present in the inflamed rat paw was powerfully inhibited by several acidic anti-inflammatory drugs.

We have made kinetic studies on the inhibition of crystalline beef α-chymotrypsin (British Drug Houses Ltd.) by some of these acidic drugs and have found that, with the exception of indomethacin (*N*-*p*-chlorbenzoyl-5-methoxy-2-methylindole-3-acetic acid) and its indol-3-yl-α-propionic analogue, they all compete with the enzyme substrate when this is *N*-acetyltyrosine ethyl ester (ATEE).

An *N*-methylene analogue of indomethacin (*N*-*p*-chlorbenzyl-5-methoxy-2-methylindol-3-yl-α-propionic acid) did, however, act as a competitive inhibitor of ATEE hydrolysis by chymotrypsin. Indomethacin itself (an *N*-acyl compound) was slowly hydrolysed by chymotrypsin to yield *p*-chlorbenzoic acid and 5-methoxy-2-methylindole-3-acetic acid.

TABLE 1. EFFECTIVENESS OF SOME ANTI-INFLAMMATORY ACIDS IN INHIBITING CHYMOTRYPTIC HYDROLYSIS OF *N*-ACETYLTYROSINE ETHYL ESTER, AND 5-HT FORMATION BY DOPA DECARBOXYLASE (BEEF ADRENAL MEDULLA)

Drug	K_I for	
	Chymotrypsin	Dopa decarboxylase
Salicylic acid	9×10^{-3}M	$1{\cdot}5 \times 10^{-2}$M
Ibufenac	5×10^{-3}M	6×10^{-4}M
Phenylbutazone	3×10^{-4}M	2×10^{-4}M
Indomethacin	No inhibition	1×10^{-4}M
Flufenamic acid	$1{\cdot}4 \times 10^{-4}$M	5×10^{-5}M
Mefenamic acid	9×10^{-5}M	4×10^{-5}M

K_I = dissociation constant of the (inhibited) enzyme-drug complex; determined graphically (Dixon, 1953).

Experimental conditions.

Chymotrypsin. Substrate concentrations 1×10^{-3}M and 6×10^{-4}M ATEE, 15 μg crystalline α-chymotrypsin all in 10 ml 10 mM sodium phosphate pH 7·50, ionic strength 0·15 (sodium chloride). Drugs and substrate added in the same buffer, enzyme added in water (30 μl 0·5 mg/ml). Reaction followed titrimetrically with a pH-stat (Radiometer, Copenhagen), using 10 mM sodium hydroxide (ionic strength 0·15).

Dopa decarboxylase. 6-fold purified enzyme from adrenal medulla. Incubation volume 0·6 ml containing 0·083 M sodium phosphate pH 6·8, 6 mM β-mercaptoethanol and 1·67 mM EDTA. Substrate concentrations 3·8 and $7{\cdot}6 \times 10^{-5}$M 5-hydroxytryptophan. 2·5 mg enzyme protein added. ^{14}C-5-HT measured after paper chromatography (Somerville, 1964).

5-Hydroxytryptamine (5-HT) has been implicated as an inflammatory mediator, at least in the rat (Spector & Willoughby, 1965). We have found that these aromatic acidic drugs also inhibit 5-HT formation by the aromatic L-amino-acid decarboxylase (dopa decarboxylase) present in bovine adrenal medulla. This decarboxylase contains strongly-bound pyridoxal phosphate and even after ammonium sulphate fractionation and gel-filtration of the enzyme, the activity of the preparation is increased only by 30% by adding excess pyridoxal phosphate (15 μM). Kinetic analysis has shown that the drugs compete with the substrate, 5-hydroxytryptophan; not with the coenzyme. [This is exactly the converse of the action of these drugs in inhibiting histamine formation by substrate-specific mammalian histidine-decarboxylases (Skidmore & Whitehouse, 1966).]

Table 1 shows the relative potencies of some acidic anti-inflammatory acids, currently used as clinical antirheumatic drugs, as competitive inhibitors of chymotrypsin and dopa decarboxylase. Glycyrrhetic acid-3-hemisuccinate (carbenoxolone 0·9 mM) and chloroquine phosphate (1·8 mM), which are examples of a non-aromatic and a non-acidic anti-inflammatory drug respectively, did not affect the chymotryptic hydrolysis of *N*-acetyl tyrosine ester or formation of 5-HT. Lauric acid (1·0 mM), which inhibits the anaphylactic release of histamine (Austen & Brocklehurst, 1961b) did not inhibit the chymotryptic hydrolysis of ATEE.

These drugs did not inhibit rat liver homogentisate oxidase (with 2 mM substrate and drugs in saturated solution, pH 7·2 or 25 mM sodium salicylate), indicating that they do not inhibit the metabolism of all phenolic derivatives of aromatic amino-acids. Unlike dopa decarboxylase and chymotrypsin, homogentisate oxidase will act only on one substrate (2,5-dihydroxyphenylacetate). It would thus seem that only enzymes with a fairly broad (binding) specificity for aromatic substrates may suffer competitive inhibition by aromatic anti-inflammatory acids. Even this viewpoint is not an exclusive one, for the action of these drugs in inhibiting histamine formation (Skidmore & Whitehouse, 1966) may be considered *either* as competitive inhibition of the binding of the aromatic cofactor (pyridoxal phosphate) *or* non-competitive inhibition of the enzyme due to drug-binding at a site not involved in binding the substrate (L-histidine).

In summary, we believe these drugs may influence several enzyme systems underlying the inflammatory response by at least two modes of action at the molecular level, which include the neutralisation of active (lysine) ϵ-amino groups as previously discussed (Whitehouse & Skidmore, 1965), and by behaving as antimetabolites to competitively inhibit enzymes such as dopa decarboxylase and (non-enteric) chymotryptic hydrolases.

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Department of Biochemistry,
University of Oxford,
South Parks Road,
Oxford.
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I. F. SKIDMORE
M. W. WHITEHOUSE*

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* Present address: Department of Experimental Pathology, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T., Australia.