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BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—X

THE INHIBITION OF SEROTONIN FORMATION *IN VITRO* AND INHIBITION OF THE ESTERASE ACTIVITY OF α-CHYMYOTRYPSIN

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Abstract—Non-steroid anti-inflammatory acids inhibit two enzymes with broad substrate-specificity for aromatic aminoacid derivatives namely 5-hydroxytryptophan decarboxylase and α-chymotrypsin, by inhibiting competitively the binding of substrate to the enzyme. Some clinically inactive analogues of these acids were much less effective as inhibitors of these enzymes. Indomethacin does not inhibit the chymotryptic hydrolysis of N-acetyltyrosine ethyl ester but may be itself hydrolysed by the enzyme.

These findings are discussed with respect to the possible role of serotonin (5-hydroxytryptamine) and of non-enteric chymotryptic enzymes as inflammatory mediators.

The correlation between clinical drug activity, physical properties and effectiveness as inhibitors of these and other enzyme systems *in vitro* is made, for salicylate, Ibufenac, phenylbutazone, indomethacin and the fenamic acids.

Concerning serotonin

IT HAS been suggested[1] that serotonin (5-hydroxytryptamine, 5-HT†) may be a mediator of the inflammatory response, being liberated from blood platelets and other tissues following injury. However, this amine may only be of importance as an inflammatory mediator in certain animal species (e.g. the rat) and then only following certain types of injury.

5-HT has been detected in the oedema fluid following induction of an experimental oedema in rats with egg-white or dextran.[2, 3] Furthermore, when 5-HT is administered (intradermally or subcutaneously) to the rat, it produces a local oedema[4] by causing vasodilatation and increasing the capillary permeability.[5]

Attempts have been made to evaluate anti-inflammatory drugs by measuring their ability to suppress the oedema caused by the cutaneous injection of 5-HT or of substances known to release 5-HT *in vivo*. However, very high levels of the conventional anti-inflammatory/anti-rheumatic drugs are needed to suppress this oedema, for example, 400–600 mg/kg sodium salicylate, 200 mg/kg phenylbutazone and

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† Abbreviations used: 5-HTP—5-hydroxytryptophan; 5-HT—5-hydroxytryptamine; ATEE—N-acetyltyrosine ethyl ester; NPA—4-nitrophenyl acetate; DMF—N,N-dimethylformamide; DOPA—3,4-Dihydroxyphenylalanine; PMSF—pheylmethane sulphonyl fluoride; 5-MeO-2-MeIAA—5-Methoxy-2-methylIndole acetic acid; 5-HIAA—5-hydroxyindole acetic acid.

200 mg/kg phenazone.[6] Known anti-5-HT drugs, such as BOL 148 (2-bromolysergic acid diethylamide) at 4 mg/kg, are much more effective in suppressing this oedema.

Two enzymes are responsible for the biosynthesis of 5-HT in mammalian tissues. These are:

1. L-Tryptophan hydroxylase, found only in those tissues which are capable of producing 5-HT, and which forms 5-hydroxytryptophan (5-HTP) from L-tryptophan.[7]

2. L-5-HTP decarboxylase, which is widely distributed in mammalian tissues and which appears to be identical with DOPA decarboxylase[8] and with L-aromatic-aminoacid decarboxylase.[9] This decarboxylase has a broad substrate-specificity and is also inhibited by a wide range of compounds, some of which are of biological and pharmacological importance.[10] We have now shown that a number of acidic anti-inflammatory compounds are moderately effective inhibitors of this 5-HTP decarboxylase.

Concerning chymotrypsin

Lagunoff and Benditt[11] have characterized an esterase ("chymase") from rat mast cell granules and shown it to be homospecific with chymotrypsin, i.e. it hydrolyses esters and amides of L-aromatic amino acids. Two possible functions of this esterase have been suggested:

1. that it is involved in the release of histamine from the granules.

2. that, when released from the granules, it mediates the increase in capillary permeability which occurs during the inflammatory response.

It is known that certain compounds with anti-esterase activity (e.g. quinine and quinidine) will partially suppress the increased vascular permeability which normally occurs during experimental inflammation.[12] It has also been shown that chymotrypsin itself can cause degranulation and concomitant release of histamine.[13, 14] Enzymes resembling chymotrypsin in their substrate-specificity have also been implicated in:

1. the swelling of a rat's paw following local injection of an inflammatory agent.[15]

2. the anaphylactic release of histamine from sensitized guinea-pig lung[16] or from rat peritoneal mast cells.[17]

Sodium salicylate is reported to inhibit a protease from guinea-pig lung[18] and also the anaphylactic release of histamine from guinea-pigs[19] and rabbits.[20] Mörsdorf and his colleagues[21] have found that the N-acetyltyrosine esterase activity of a homogenate of inflamed rat paw is inhibited by low concentrations of salicylate and other acidic anti-inflammatory drugs.

We have now studied the esterase activity of bovine α-chymotrypsin as a model for the (non-enteric) chymotryptic esterases of inflammation and found that aromatic acidic anti-inflammatory drugs are competitive inhibitors of the enzyme when acetyltyrosine ethyl ester (ATEE) is the substrate.

This work has been the subject of a preliminary communication.[22]

MATERIALS

DL-5-hydroxytryptophan (methylene-C-14) specific activity 21·8 mc/m-mole (99 μc/mg) was obtained from the Radiochemical Centre, Amersham, Bucks., DL-5-HTP and α-methyl DOPA were obtained from the Sigma Chemical Co., London. Bovine α-chymotrypsin, four times recrystallized, N-acetyl tyrosine ethyl ester and

4-nitrophenyl acetate (NPA) were obtained from British Drug Houses Ltd., Poole, Dorset. The sources of the anti-inflammatory drugs used are detailed elsewhere.[23]

METHODS

Preparation of the decarboxylase

Bovine adrenals were obtained from the local slaughterhouse and were brought to the laboratory on ice. The medullary tissue was dissected at once and either used immediately or stored at −15° until required. The tissue retained its enzymic activity when frozen for several months. 145 g of chopped tissue was homogenized in a blendor with 400 ml 0·1 M sodium phosphate, pH 6·8, containing 7·2 mM β-mercaptoethanol and 2 mM EDTA, at 2°. This homogenate was then centrifuged at 35,000 *g* for 15 min to remove most of the insoluble matter and the supernatant fluid from this centrifugation was decanted and recentrifuged at 55,000 *g* for 30 min. The resulting supernatant solution was then fractionally precipitated at 2° using saturated ammonium sulphate solution adjusted to pH 6·8. Protein fractions precipitating the ranges 0–30 per cent saturation, 30–45 per cent saturation and 45–60 per cent saturation were collected by centrifugation at 35,000 *g* for 5 min, and were redissolved in a minimum volume of 0·1 M sodium phosphate, pH 6·8 containing β-mercaptoethanol and EDTA. These solutions were desalted by passage through a column of Sephadex G-25 (2·5 × 50 cm) equilibrated with the same buffer at 2°. The desalted 30–45 per cent saturation fraction was used in all experiments without further purification. This protein solution was stable at −15° in the presence of 7·2 mM mercaptoethanol and 2 mM EDTA.

Assay of 5-HTP decarboxylase activity

The chromatographic method of Somerville[24] was used, with Whatman 3 MM paper. Incubations were carried out at 37° in a "Sero-Block" (Precision Instruments, Chicago) using a final volume of 0·6 ml. The incubation mixtures were made up as follows:

0·1 ml ^{14}C-5-HTP (0·5 μc, 2·3 × 10^{-4}M) in 0·01N HCl.
0·1 ml cold 5-HTP (2·3 × 10^{-4}M) in buffer, pH 6·8.
0·1 ml protein solution (25 mg/ml).
0·1–0·3 ml drug solution in the same buffer.
buffer as above to 0·6 ml.

The protein solution (at 37°) was added last to the incubation mixture and contained, under normal assay conditions, no added pyridoxal phosphate. Controls with heat-denatured enzyme and drug-free controls were analysed simultaneously with the incubations containing drugs. Ten microlitre samples were taken for analysis at the beginning and throughout the incubation. Radioactivity of the chromatogram was measured as described previously.[23]

Hydrolysis of N-acetyltyrosine ethyl ester

This was measured by the continuous titration of protons liberated during hydrolysis, using a pH stat (Radiometer, Copenhagen). Hydrolysis was carried out at 20 ± 2° and at pH 7·5 in 0·01 M sodium phosphate, adjusted to ionic strength 0·15 with sodium chloride. The substrate also was dissolved in this buffer to give a 10 mM solution. All drugs were dissolved in this buffer and, where necessary, were adjusted to pH 7·5 with sodium hydroxide (0·1 or 1·0 N). The titrating solution was 0·01 N

sodium hydroxide (ionic strength 0·15). Crystalline α-chymotrypsin was dissolved in water to give a solution of 0·5 mg/ml which was stored in ice (enzyme activity was unchanged for up to 3 hr if kept under these conditions).

Incubation mixtures (total volume 10 ml) contained from 0·1 to 2 mM substrate and varying concentrations of drugs. After the pH of the solution was stabilized at pH 7·5, 30 μl (15 μg) of enzyme were added and the titration followed on the recorder. Control experiments in the absence of drug and in the absence of enzyme were also recorded. The initial rate of hydrolysis was calculated from the recorder tracing.

Hydrolysis of 4-nitrophenyl acetate

This was followed spectrophotometrically at pH 7·5 and 20 ± 2° by the increase in extinction at 400 mμ. Two sets of experiments were carried out.

1. For following the initial acetylation of the enzyme, the initial rapid rate of nitrophenol liberation was recorded on a Zeiss PMQ II spectrophotometer attached to a Sargent potentiometric recorder geared to record extinction. A reaction volume of 3·0 ml was used, made up as follows: 2·9 ml 0·1 M sodium phosphate pH 7·5, or drug solution in the same buffer was mixed with 0·1 ml of enzyme solution (30 mg/ml in water). This solution was placed in a cuvette (1 cm light path) and the change in extinction with time, 400 mμ, recorded using a chart speed of 4 in/sec and with a reference cell containing buffer alone. The sample compartment was then opened (this closes the shutter) and 20 μl of substrate solution was added on the end of a glass stirrer and the solution mixed rapidly. The initial concentration of the substrate was 9×10^{-3} dissolved in *iso*propanol, giving a final substrate concentration of 6×10^{-5}M. After mixing the compartment was shut and reading continued as the shutter opened. The time taken to add the substrate and close the compartment was less than 3 sec and recording was continued for at least 2 min after mixing. Experiments without enzyme showed that, at this substrate concentration there was no measurable increase in extinction due to non-enzymic hydrolysis of the substrate.

2. For following the rate of NPA hydrolysis a reaction mixture was made up as follows: 4·5 ml 0·1 M sodium phosphate, pH 7·5 (containing drugs in some experiments) was mixed with 0·25 ml 6×10^{-2}M NPA in *iso*propanol giving a final substrate concentration of 3×10^{-3}M. A sample of this solution was placed in the spectrophotometer (Hilger-Watt Uvispek) and the change in extinction with time recorded, this gave the rate of non-enzymic hydrolysis, which at this substrate concentration was appreciable. This solution was then returned to the reaction mixture and 0·25 ml of enzyme solution (5 mg/ml in water) was added and mixed. A sample was again taken and the extinction at 400 mμ recorded at 30-sec intervals. This rate of change of extinction was corrected for the non-enzymic hydrolysis to obtain the true rate of enzymic hydrolysis.

In both types of experiments using nitrophenyl acetate as a substrate, the drug solutions contained a small amount of N,N-dimethylformamide so that the final concentration of DMF was 0·5 per cent by volume. The same concentration of DMF was present in drug-free controls.

The hydrolysis of indomethacin by chymotrypsin

Indomethacin, being an N-acyl compound, is a possible substrate for chymotrypsin (hydrolysis would yield *p* chlorobenzoic acid and 5-methoxy-2-methylindole-3-acetic

acid). Indomethacin therefore was incubated with a large amount of chymotrypsin and the reaction products were analysed by thin-layer chromatography. Chymotrypsin, at a final concentration of 13 mg/ml, was incubated with 1·5 mM indomethacin in the presence and absence of the inhibitor phenylmethane sulphonylfluoride (PMSF)[25] for 2 hr at 37° in the dark. The reaction mixture was then adjusted to pH 2 with 6 M perchloric acid and the precipitated protein removed by centrifugation. The acidic supernatant solution was then extracted with two 5-ml portions of ethyl acetate and the extracts for each incubation were combined and evaporated under nitrogen in the dark. Both indomethacin and 5-methoxy-2-methylindole-3-acetic acid (5-MeO-2-Me-IAA) were put through the same extraction procedure. The residues were then dissolved in 25 μl ethanol and 10 μl of this solution was streaked on a 20 × 20 cm thin layer plate of Silicagel H (E. Merck, Darmstadt). The plate was developed in the dark using a solvent system, methyl acetate; *iso*propanol; 25% ammonia (9:7:4 by volume).[26] Authentic samples of indomethacin and 5-methoxy-2-methylindole-3-acetic acid were developed on the same plate. Compounds were located under u.v. light.

RESULTS

Properties of 5-*HTP decarboxylase*

In agreement with the findings of Fellman[27] most of the enzymic activity was precipitated between 30 and 45 per cent saturation with ammonium sulphate at pH 6·8. This protein fraction contained between 9·5 and 13 per cent of the total protein of the initial 55,000 *g* supernatant and from 64 to 69 per cent of the activity of this fraction, representing a six-fold purification. This preparation was used without further purification. Fellman obtained a homogeneous protein after an eighteen-fold purification.

The activity of the preparation varied only slightly with the length of time the tissue had been stored at −15° before use. A typical preparation from tissue which had been stored for 4 months had an activity of 10·8 mμmoles substrate converted per mg protein per hr at pH 6·8 and 37° with a substrate concentration of $3{\cdot}8 \times 10^{-5}$M and without any added pyridoxal phosphate. (These are not optimal conditions of pH or substrate concentration). Fresh preparations were up to 20 per cent more active. If pyridoxal phosphate was added to the incubation mixture at a concentration of $1{\cdot}5 \times 10^{-5}$M the activity of the enzyme increased by about 35 per cent indicating that perhaps the enzyme was about 30 per cent resolved from its cofactor. Further gel-filtration of the enzyme did not increase the dissociation of the enzyme-substrate complex.

The presence of both EDTA and mercaptoethanol in the incubation mixture increased the enzyme activity by a maximum of 15–20 per cent and they were therefore routinely included in all incubations. The final concentration of mercaptoethanol was 6 mM and of EDTA 1·67 mM.

It was found that the rate of decarboxylation was linear with time until at least 15 per cent of the radioactive (DL) substrate had been decarboxylated; this corresponds to a 30 per cent conversion of the true substrate, L-5-hydroxytryptophan. Usually experiments were designed to give a yield of 5-HT corresponding to a 10–12 per cent conversion of substrate, as this enabled the amount of amine formed to be determined with accuracy, even in the presence of considerable concentrations of inhibitor.

Incubation times therefore varied from 8 to 20 min, depending on the initial activity of the enzyme preparation. The enzyme remained stable at 37° for such periods.

Km for DL-5-HTP was found to be $1{\cdot}3 \times 10^{-4}$M at pH 6·8 and 37°. Yuwiler *et al.*[28] reported a Km for hog kidney 5-HTP decarboxylase of $1{\cdot}7 \times 10^{-4}$M at pH 6·8 in the same buffer. The enzyme was inhibited completely by 1×10^{-4}M α-methyl DOPA and by N-ethyl maleimide (in the absence of mercaptoethanol) at 1×10^{-4}M.

The effects of anti-inflammatory acids and their analogues on the decarboxylation of 5-HTP

Table 1 shows the effect of some acidic anti-inflammatory drugs and some of their chemical analogues on the decarboxylation of 5-HTP by the partially purified aro-

TABLE 1. COMPARISON OF THE INHIBITION OF 5-HTP DECARBOXYLASE BY SOME ANTI-INFLAMMATORY DRUGS AND THEIR PHARMACOLOGICALLY INACTIVE ANALOGUES

Drug	Conc. mM	% Inhibition	Analogue	Conc. mM	% Inhibition
Sodium salicylate	1·0	23	4-hydroxybenzoate	1·0	0
Ibufenac	1·0	40	Phenylacetic acid	1·0	13
Phenylbutazone	0·15	20	Phenazone	1·0	12
			Amidopyrine	1·0	0
Indomethacin	0·10	32	5-methoxyindole-3-acetic acid	1·0	29
5-Fluoro analogue of indomethacin	0·05	52			
Mefenamic acid	0·05	50	N-methyl anthranilic acid	1·0	39
Flufenamic acid	0·05	33			

Substrate concentration $3{\cdot}8 \times 10^{-5}$M 5-HTP, 2·5 mg enzyme in 0·6 ml reaction volume, pH 6·8, 37°. Percentage inhibition calculated as the percentage decrease in the conversion of substrate from drug-free controls.

matic aminoacid decarboxylase from adrenal medulla. The clinically active drugs appear to be from ten to twenty times more effective as inhibitors than their inactive (and sometimes non-acidic) analogues. Even the acidic analogues are much less active than the clinical drugs.

Table 2 gives Ki values for the active anti-inflammatory drugs and also the type of inhibition shown with respect to the substrate. Their effectiveness as inhibitors of 5-HTP decarboxylase actually parallels their inhibition of substrate specific histidine decarboxylase of foetal rat tissue,[23] i.e. salicylate < Ibufenac < phenylbutazone < indomethacin < flufenamic acid. The Ki's for a given drug for the two enzymes are of the same order. However, with 5-HTP decarboxylase the inhibition is competitive with respect to the substrate, so that there is competition between substrate and inhibitor for a site on the enzyme, rather than competition between cofactor and drug as is the case with histidine decarboxylase.

Sanochrysin (sodium aurothiosulphate) and Biogastrone (Carbenoxolone. 18β-glycyrrhetic acid hemisuccinate) which are acidic and non-aromatic anti-inflammatory compounds, did not inhibit 5-HTP decarboxylase when tested at a concentration of 1 mM (these compounds do inhibit histidine decarboxylase[23]).

TABLE 2. CHARACTERISTICS OF THE INHIBITION OF 5-HTP DECARBOXYLASE BY SOME ACIDIC ANTI-INFLAMMATORY DRUGS

Drug	Ki mM	Type of inhibition relative to substrate
Sodium salicylate	1·5	Competitive
Ibufenac	0·6	,,
Phenylbutazone	0·2	,,
Indomethacin	0·1	,,
5-Fluoroindomethacin	0·04	,,
Mefenamic acid	0·04	,,
Flufenamic acid	0·05	,,

Incubation conditions. Substrate concentrations 3·8 and $7{\cdot}6 \times 10^{-5}$M 5-HTP, pH 6·8 at 37°. Mode of inhibition and Ki's determined graphically by the method of Dixon.[29]

The hydrolysis of ATEE by chymotrypsin and the effect of anti-inflammatory drugs on this esterase activity

It was found that the Km for ATEE was 9×10^{-4}M under the conditions of the experiments (pH 7·5, ionic strength 0·15, 20°). Table 3 shows that, with the exception of indomethacin the acidic anti-inflammatory drugs inhibit the hydrolysis of ATEE by chymotrypsin and that their inhibition is competitive with respect to substrate. However, this pancreatic enzyme is less sensitive to these drugs than is either 5-HTP decarbxoylase or the substrate specific histidine decarboxylase.[23]

TABLE 3. CHARACTERISTICS OF THE INHIBITION OF THE HYDROLYSIS OF N-ACETYL TYROSINE ETHYL ESTER BY CHYMOTRYPSIN

Drug	Ki mM	Type of inhibition relative to substrate
Sodium salicylate	9·0	Competitive
Ibufenac	5·0	,,
Phenylbutazone	0·3	,,
Indomethacin	Not an inhibitor	—
Flufenamic acid	0·14	Competitive
Mefenamic acid	0·08	,,
N-*Benzyl* analogue* of Indomethacin	0·5	,,
N-*Benzoyl* analogue*	Not an inhibitor	—

Substrate concentration 1·0 and $0{\cdot}6 \times 10^{-3}$M ATEE, pH 7·5 (0·01 M sodium phosphate, ionic strength 0·15). Temperature 20°.
* These were the corresponding indol-3-yl-α-propionic acids.

Neither indomethacin itself, (1-(*p* chlorbenzoyl)-5-methoxy-2-methylindole-3-acetic acid) nor its propionyl analogue, 1-(*p* chlorbenzoyl)-5-methoxy-2-methylindole-3-yl-α-propionic acid, inhibited this esterase activity, even at low substrate concentrations and with high drug concentrations. However, the *benzyl*-α-propionyl analogue, 1-(*p* chlor*benzyl*)-5-methoxy-2-methylindol-3-yl-acetic acid, did inhibit ATEE hydrolysis competitively, with a Ki of approximately 5×10^{-4}M. It was found that the clinically inactive acidic and non-acidic analogues of these drugs at 1 mM had no effect on the enzyme. Sanochrysin and biogastrone had no effect at this concentration

either, nor did the basic drug, chloroquine phosphate at 1·8 mM. 4-Nitrophenyl acetate, at a concentration of 6×10^{-5}M inhibited the hydroylsis of ATEE completely.

The initial reaction of nitrophenyl-acetate (NPA) and chymotrypsin

When chymotrypsin and NPA are mixed there is an immediate and rapid release of nitrophenol equivalent to the amount of enzyme present. This initial release of nitrophenol is due to the acetylation of the enzyme by the substrate and the subsequent steady and slower release of nitrophenol is the true enzymic reaction i.e. the hydrolysis of the substrate, which is governed by the rate at which the acetyl-chymotrypsin is hydrolysed.[30,31] The effect of anti-inflammatory drugs on this reaction was to slow down the rate of chymotrypsin acylation. This can be seen in Table 4 where the results show that phenylbutazone, flufenamic acid and mefenamic acid are all approximately as effective as each other and rather more effective than indomethacin.

TABLE 4. THE EFFECT OF ACIDIC ANTI-INFLAMMATORY DRUGS ON THE ACYLATION OF CHYMOTRYPSIN BY NITROPHENYL ACETATE

Drug	Conc. mM	% Inhibition
Phenylbutazone	0·5	25
	1·0	51
Flufenamic acid	0·5	22
	1·0	49
Mefenamic acid	0·5	28
	1·0	57
Indomethacin	1·0	32
	2·0	44
	2·7	46

Substrate concentration 6×10^{-5}M at pH 7·5. Final isopropanol concentration not greater than 0·67% by volume. Temperature 20°. Light path 1 cm. Percentage inhibition taken as the percentage decrease in the initial rate of change in extinction at 400 mμ.

The hydrolysis of nitrophenyl acetate by chymotrypsin

The results from these experiments showed that only indomethacin and its *benzoyl-α*-propionic acid analogue are inhibitors of the true enzymic hydrolysis (as measured by the continuous release of nitrophenol following the initial rapid burst of nitrophenol). It was difficult to obtain accurate values for the inhibition of NPA hydrolysis by indomethacin because the drug has considerable absorption in the region of 400 mμ. However, the results obtained, together with information gathered from the later parts of experiments on enzyme acylation are summarized in Table 5.

The hydrolysis of indomethacin by chymotrypsin

Figure 1 shows the results of a thin-layer chomratographic analysis of the products of the reaction between indomethacin and chymotrypsin in the presence and absence of the enzyme inhibitor, phenylmethane sulphonylfluoride. Identical results were obtained from duplicate experiments. It is apparent that indomethacin can act as a substrate for chymotrypsin but the actual enzymic "hydrolysis" may only amount to

the acylation of the active site of the enzyme and deacylation may not occur until the enzyme is denatured by acid. Attempts to follow the hydrolysis of indomethacin by titration were unsuccessful and the solutions absorbed too much light for it to be possible to follow any possible spectral changes.

TABLE 5. INHIBITION OF THE HYDROLYSIS OF NITROPHENYL ACETATE

Drug	Conc. mM	% Inhibition of the hydrolysis of NPA
Phenylbutazone	2·7	0
Mefenamic acid	2·7*	0
Flufenamic acid	2·7	0
Indomethacin	2·7	20
Benzoyl-α-propionyl analogue of indomethacis	2·7	8

* Nitrophenyl acetate concentration 3 mM, 0·25 mg/ml chymotrypsin, pH 7·5. 20°.

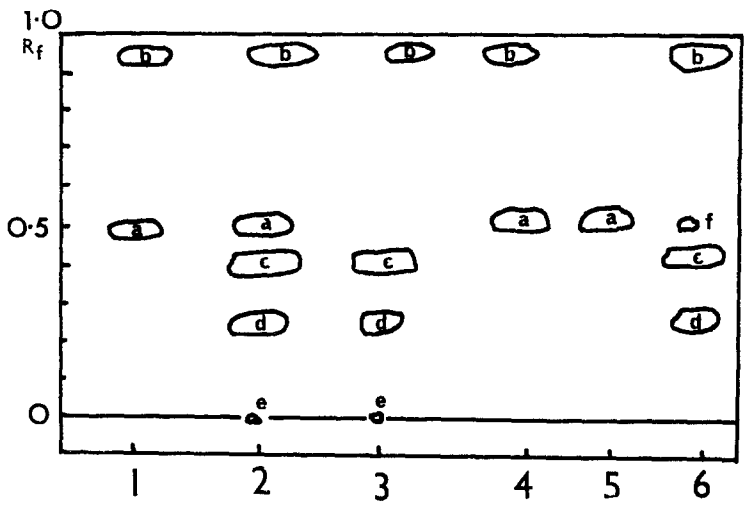

FIG. 1. Thin layer chromatogram of the products of the reaction between indomethacin and chymotrypsin.

Concentration of indomethacin 1·5 mM, chymotrypsin 13 mg/ml, incubation 2 hr at 37° in the dark

1. Chymotrypsin + PMSF + indomethacin.
2. Chymotrypsin + indomethacin.
3. 5-MeO-2-MeIAA } Both put through the full extraction procedure.
4. Indomethacin }
5. Indomethacin.
6. 5-MeO-2-MeIAA.

Characteristics of spots under u.v. light.

a. Absorbing spot R_f p·52.
b. Orange fluorescence R_f 0·95.
c. Yellow spot with blue fluorescence turning to yellow fluorescence in 12 hr R_f 0·42.
d. Blue fluorescence R_f 0·25.
e. Orange fluorescence at the origin.
f. Orange fluorescence R_f 0·5.

DISCUSSION

The decarboxylase and its assay

Because 5-HTP decarboxylase appears to be identical with DOPA decarboxylase and with L-aromatic aminoacid decarboxylase it is not surprising that the enzyme is found in tissues such as adrenal medulla, where 5-HT is not formed, or in liver and kidney, where the 5-HT levels are very low in comparison with the enzymic activity. Adrenal medulla was used in these experiments as it was a convenient source of the enzyme which needed little purification and was available in quite large quantities.

The enzymic activity, when calculated for optimal substrate conditions at pH 6·8 was found to be of the same order as that found by Fellman[27] and by Yuwiler *et al.*[32] (using hog kidney) and the Km value at this pH was similar to that obtained with this other tissue.

The method for assaying for 5-HTP decarboxylase activity appears to be reliable and accurate, giving reproducible results in duplicate incubations and in separate experiments. It is also considerably more rapid than the solvent extraction/fluorescence method of Bogdanski *et al.*[33] and is of a comparable order of sensitivity. However, because a small incubation volume is used, it is desirable to employ an enzyme preparation which is at least partially purified, in order to minimize the effects of the binding of substrate and inhibitor to non-enzymic protein. The data in Table 6 illustrates the

TABLE 6. COMPARISON OF Ki VALUES WITH CRUDE AND PARTIALLY PURIFIED DECARBOXYLASE

Drug	Crude supernatant Ki mM (1)	30–45% Saturation fraction Ki mM (1)
Indomethacin	0·35	0·1
5 Fluoroindomethacin	0·15	0·04
Flufenamic Acid	0·16	0·05
Mefenamic Acid	0·15	0·04

Experimental conditions as in Table 2.

effect of non-enzymic protein, on Ki for various acidic drugs. The effect of this extra protein in reducing the free concentration of both substrate and inhibitor is at a maximum in a small incubation volume using a crude supernatant solution. Adrenal medulla, which contains a high proportion of the decarboxylase is therefore easier to use than a preparation from liver, which would contain a higher proportion of non-enzymic protein. However, it is still necessary to purify the adrenal extract to some extent.

The effects of anti-inflammatory drugs on the enzymic activity

Although these acidic drugs are not amino-acids they act as competitive inhibitors of the decarboxylase. Accepted inhibitors of 5-HTP decarboxylase would appear to be of three types.[10, 34]

1. Substrate analogues with inhibitory activity, e.g. L-phenylalanine, noradrenaline, carboxychalcones and α-methyl DOPA. All the compounds in this group appear to be aromatic and nearly all are acids.

2. Compounds containing a reactive amino group, e.g. hydroxylamine and semicarbazide, which will react with the carbonyl group of the cofactor, pyridoxal phosphate, and which do not compete with the substrate.

3. Sulphydryl reagents, such as N-ethylmaleimide, which react with the sulphydryl group at the active site of the enzyme.

The acidic anti-inflammatory drugs which have been studied in this work appear to belong to the first of these classes, being acid and aromatic in character and also being competitive inhibitors. As both the substrate and the inhibitors of the enzyme are acidic it seems reasonable to suggest that the drugs interact with that (cationic?) group on the protein with which the acid function of the substrate normally associates. As the inhibitors do not have an α-amino group they cannot react with the cofactor and as the inhibition is competitive with respect to the substrate rather than with respect to the cofactor it seems unlikely that the drugs inhibit 5-HTP decarboxylase in the same way as they inhibit histidine decarboxylase. In the case of 5-HTP decarboxylase, although the purification procedure appears to resolve the enzyme-cofactor complex to the extent of about 30 per cent, the remaining cofactor is bound firmly to the enzyme and cannot be removed by prolonged dialysis. Therefore it is unlikely that anything other than a compound reacting with the cofactor carbonyl group, such as hydroxylamine or excess substrate,[35] would be able to displace the cofactor from the enzyme. However, as these anti-inflammatory drugs are acids they may react with another cationic group (other than that binding the cofactor) which may be responsible for binding with the acidic function of the substrate to the ezyme.

In this connexion it is probably significant that the order of effectiveness of these drugs in inhibiting the decarboxylase is the order of the extent of their binding to bovine plasma albumin[23] and in particular to the lysyl ε-amino groups of this protein.

The hydrophobic nature of these drugs in relation to their acidic analogues (4-hydroxybenzoate, N.methylanthranilate, 5-methoxyindole acetic acid) must also be important as these analogues are by no means such effective inhibitors. Similarly the non-acidic analogues of phenylbutazone are by no means as active as the drug itself. The acidic but non-aromatic anti-inflammatory drugs presumably cannot bind to the enzyme in the same way as do the aromatic drugs.

The relationship of the results in vitro *to the behaviour of these drugs* in vivo

For these above results to have real significance it must be possible to demonstrate that:

1. these drugs lower the rate of 5-HT production *in vivo*,

2. 5-HT is involved in the inflammatory response. On the other hand, these results could still be significant indirectly if those physico-chemical properties of the drugs which make them effective inhibitors of 5-HTP decarboxylase *in vitro* are the same properties which make them effective anti-inflammatory drugs *in vivo*.

It would be instructive to have adequate data on the effects of these drugs on the tissue levels of 5-HT as this would give some idea of the effect on 5-HT metabolism as a whole. Another approach would be to measure the excretion of 5-HT and its metabolites (mainly 5-hydroxyindole acetic acid, 5-HIAA) in the urine. Here some progress has been made as there are reports that phenylketonurics have lowered 5-HIAA levels and raised phenylpyruvic, phenyl-lactic and phenylacetic acid levels in the urine and lowered 5-HT levels in the blood.[36, 37] When these patients are given

phenylalanine-free diets the levels of the phenylalanine metabolites drop and the levels of 5-HT in the blood and 5-HIAA in the urine rise. These phenylalanine metabolites are inhibitors of 5-HTP decarboxylase *in vitro*[38] and it is suggested that they are functioning as such during phenylketonuria and lowering the rate of synthesis of 5-HT *in vivo*.

Another approach would be to study the pools of "stored" and "free" 5-HT. It is generally considered that 5-HTP decarboxylase inhibitors can have little effect on the levels of 5-HT in the body under normal conditions, as the decarboxylase activity is very much greater than the tryptophan hydroxylase activity. However, it has been pointed out that the monoamines of the tissues are probably localized into "stored" and "free" pools in different parts of the tissue.[39] Under normal conditions the "free" 5-HT would only be a small proportion of the total 5-HT and even though it were turning over quite rapidly, inhibition of the turnover of this fraction would have little or no effect on the rate of turnover of the total body 5-HT. A method such as has recently been developed for the differential labelling of the free histamine pool[40] would be invaluable in such circumstances.

The inhibition of the esterase activity of α-chymotrypsin

Foster[41] assumed that the reaction of chymotrypsin with substrate followed the

$$\mathrm{EH} + \mathrm{AcB} \overset{1\cdot1}{\rightleftharpoons} (\mathrm{EH.AcB}) \overset{1\cdot2}{\rightleftharpoons} (\mathrm{EAC.HB}) \overset{1\cdot3}{\rightleftharpoons} \mathrm{EAC} + \mathrm{HB}$$

$$\mathrm{EAc} + \mathrm{HOR} \overset{2\cdot3}{\rightleftharpoons} (\mathrm{EAc.HOR}) \overset{2\cdot2}{\rightleftharpoons} (\mathrm{EH.AcOR}) \overset{2\cdot1}{\rightleftharpoons} \mathrm{EH} + \mathrm{AcOR}$$

in which EH is the enzyme and EAc is the acetyl-enzyme complex. The quantities in brackets are the appropriate complexes. Although Foster wrote this equation for the hydrolysis of nitrophenyl acetate it can be applied to other typical ester substrates.

Foster found also that substances which were inhibitors of the overall reaction with a good substrate (such as ATEE, with a low Km and a fast overall rate of hydrolysis) inhibited only the initial acylation when NPA was used as the substrate. He suggested that the most probable site for this inhibition was the initial binding of the substrate to the enzyme, that is, at reaction 1·1.

The results obtained with salicylic acid, phenylbutazone and the fenamic acids are similar in that the hydrolysis of ATEE and only the initial acylation by NPA are inhibited. It is therefore reasonable to assume that a similar mechanism of inhibition is in operation. However, the results with indomethacin and its analogues are more difficult to interpret as indomethacin can inhibit the initial acylation of the enzyme by NPA and also the true hydrolysis of this substrate but is unable to inhibit the hydrolysis of ATEE. The answer may lie in the fact that indomethacin is an N-acyl compound and as such can act as a substrate, or at least as a source of acyl groups for the active site of the enzyme. The studies on the hydrolysis of indomethacin show that 5-methoxy-2-methylindole-3-acetic acid is liberated during the reaction but there is no evidence whether this is a true enzymic hydrolysis or whether the enzyme just becomes acylated and is unable to deacylate itself.

If indomethacin and the substrate compete for the serine hydroxyl group at the active site of the enzyme rather than for the initial binding site of the substrate and if the rate-limiting step of the ATEE hydrolysis is the initial binding rather than the

TABLE 7. BIOCHEMICAL AND PHYSICO-CHEMICAL PROPERTIES OF SOME ACIDIC ANTI-INFLAMMATORY COMPOUNDS

Drug	Clinical dose g/day	Therapeutic conc. in blood	Conc. for 50 per cent inhibition oxidative phosphorylation	Conc. for 50 per cent inhibition ATEE esterase[42]	Ki Histidine decarb-oxylase[23]	Ki 5-HTP decarboxylase	Ki Chymotrypsin	pK_a	Partition coeff. *N*-octanol :water pH 7·5	Binding to albumin μmoles/ μmole albumin pH 7·5[c]	Per cent inhibition binding of TNBAL to albumin[23]
Sodium salicylate	2+	1×10^{-3}M	8×10^{-4}M	$1{\cdot}6 \times 10^{-3}$M	$1{\cdot}2 \times 10^{-3}$M	$1{\cdot}5 \times 10^{-3}$M	9×10^{-3}M	3·0	0·06	1·7	31
Ibufenac	2·4	$\sim 1 \times 10^{-3}$M	1×10^{-3}M	—	8×10^{-4}M	6×10^{-4}M	5×10^{-3}M	~4·2	—	—	47
Phenylbutazone	0·3	$2{\cdot}5 \times 10^{-4}$M	2×10^{-4}M	$6{\cdot}4 \times 10^{-4}$M	4×10^{-4}M	3×10^{-4}M	3×10^{-4}M	4·5	3·9	2·4	55
Indomethacin	0·2	—	2×10^{-4}M	$3{\cdot}3 \times 10^{-5}$M	2×10^{-4}M	1×10^{-4}M	—	~5·3[a]	8·5	3·1	75
Flufenamic acid	0·3	—	$<1 \times 10^{-4}$M	$3{\cdot}6 \times 10^{-5}$M	3×10^{-5}M	5×10^{-5}M	$1{\cdot}4 \times 10^{-4}$M	5·5	15	4·0	74
Mefenamic acid	0·3	—	1×10^{-4}M	$1{\cdot}4 \times 10^{-5}$M	—	4×10^{-5}M	8×10^{-5}M	6·6[b]	15	—	57

[a] Measured in 50% methyl cellosolve.
[b] Measured in 75% ethanol.
[c] Skidmore & Whitehouse, unpublished results.

acylation of the serine hydroxyl, then indomethacin would not be able to inhibit the hydrolysis of ATEE (nor would its *benzoyl*-propionyl analogue, another N-acyl compound). Its *benzyl*-propionic analogue, not being an N-acyl compound, would perhaps compete for the substrate binding site rather than for the serine hydroxyl group of the active centre of the enzyme.

In the case of NPA hydrolysis, if indomethacin does acylate some of the enzyme then this means that the amount of enzyme available for acetylation by NPA will be lower and therefore the rate of deacylation will also be lower. As deacylation of acetyl-chymotrypsin is the rate-limiting step of the hydrolysis of NPA this will result in a fall in the rate of hydrolysis of NPA. As NPA is a poor substrate with a high Km and a low rate of hydrolysis, and as the rate of enzymic acetylation is measurable, it is possible that the rate of acetylation is governed by reaction 1·2 or 1·3 rather than by 1·1 with the result that indomethacin could also have an inhibitory effect on the initial acylation of the enzyme by NPA.

Domenjoz[42] quotes some data from Mörsdorf's experiments which show that the ATEE esterase from inflamed tissue is far more sensitive to acidic anti-inflammatory drugs than is bovine α-chymotrypsin. Also this enzyme, from rat paw, is more sensitive to these drugs than any other enzyme so far tested. Indomethacin apparently inhibits this esterase even though it does not inhibit the ATEE esterase activity of chymotrypsin.

GENERAL CONCLUSIONS

The relative effectiveness of current clinically active non-steroidal anti-rheumatic drugs (indicated by their daily requirement) closely follows

1. their relative effectiveness as inhibitors of the following enzyme systems
   a. Mitochondrial energy conservation (oxidative phosphorylation),
   b. (Pro-inflammatory) amino-acid decarboxylases,
   c. Chymotryptic esterases.

and 2. their relative inhibition of the binding of aromatic aldehydes (pyridoxal phosphate and trinitrobenzaldehyde) to albumin amino groups and the relative binding of the drugs themselves to plasma albumin. (Table 7).

Drug effectiveness also increases in this series of compounds with increase in p*Ka* (provided the drug remains an acid) and with increasing lipophilic (hydrophobic) character.

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