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BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—VIII

INHIBITION OF HISTAMINE FORMATION CATALYSED BY SUBSTRATE-SPECIFIC MAMMALIAN HISTIDINE DECARBOXYLASES. DRUG ANTAGONISM OF ALDEHYDE BINDING TO PROTEIN AMINO GROUPS

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Abstract—Kinetic studies have shown that a number of acidic anti-inflammatory drugs (salicylate, "Ibufenac", phenylbutazone, Indomethacin and Flufenamic acid) inhibit histamine formation by competing with pyridoxal phosphate for the coenzyme binding site, believed to be a lysyl ε-amino group on mammalian histidine decarboxylase. Apparent inhibitor constants (*Ki*'s) for these drugs were measured.

These drugs also inhibited the binding of pyridoxal phosphate and 2,4,6-trinitrobenzaldehyde (TNBAL) to ε-amino groups of bovine plasma albumin at pH 7·5.

A number of chemical analogues of these drugs were screened for their ability to (i) inhibit histamine formation *in vitro* and to (ii) retard the formation of the red coloration which forms when TNBAL reacts with albumin at pH 7·5 (and provides a novel colorimetric method for testing potential drug activity).

Other compounds with notable drug activity in these two assays [(i) and (ii) above] include Sanochrysin, "Biogastrone", pentachlorophenol and phenylindanedione—all of which, like the anti-inflammatory drugs named above, also uncouple oxidative phosphorylation.

EVER since Sir Thomas Lewis[1] showed that the triple response in skin could be elicited with histamine, this amine has been repeatedly implicated as a mediator of the early inflammatory reponse. This early response is due to the release of pre-formed histamine stored in the tissues and is antagonized by many basic drugs, the so-called "antihistamines".

Schayer[2, 3] has recently presented a theory that histamine is continually generated in the tissues as a chemical mediator of microcirculatory changes, and that the anti-inflammatory glucorticoids act as inhibitory regulators of histamine-mediated permeability changes in the microcirculation. Kahlson and his colleagues[4–7] have drawn attention to the association between histamine formation and tissue proliferation in various animal tissues. These recent studies therefore suggest that histamine may play a further role in inflammation and wound healing, in addition to initiating the triple response. We have now found that many non-steroidal anti-inflammatory drugs are potent inhibitors of histamine formation *in vitro* by enzymes present in animal tissues.

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Smith and his co-workers[8–10] found that the decarboxylation of L-glutamic acid by an enzyme from *E. coli* and rat brain was inhibited by relatively high levels of compounds which were congeners of salicylic acid, having a thiol or hydroxyl group *ortho* to the carboxyl group. They showed that inhibition by such drugs was reduced by pre-incubation of the enzyme with pyridoxal phosphate (PXALP)* but complete reversal of the inhibition was never achieved in this manner. Increasing the substrate concentration had no effect upon the degree of inhibition.

Amino acid decarboxylases require PXALP as a cofactor. Other PXALP-dependent enzymes, e.g. serum and tissue transaminases, have also been found to be sensitive to salicylates[11, 12] and other acidic anti-inflammatory drugs such as phenylbutazone and cinchophene.[13–15]

There are at least two classes of enzymes, in mammalian tissues, which decarboxylate histidine:

(i) those which are specific for histidine as a substrate, and

(ii) the relatively non-specific L-aromatic amino acid decarboxylases. The enzyme(s) principally studied in this work fall into the first of these classes and, unlike many other amino acid decarboxylases, they bind PXALP rather weakly.[16] It would appear that the relative potency of an anti-inflammatory drug in inhibiting histamine formation *in vitro* parallels its ability to displace PXALP from the enzyme protein.

In order to estimate the ability of a drug to displace PXALP from the enzyme protein we have devised two assays based on a spectrophotometric estimation of the binding of PXALP and 2,4,6-trinitrobenzaldehyde respectively to bovine plasma albumin. Preliminary accounts of this work have appeared elsewhere.[17, 18]

MATERIALS

Materials were obtained from the following sources: L-histidine (ring-2-C14) 35·0 mc/m-mole from the Radiochemical Centre, Amersham, Bucks; pyridoxal-5′-phosphoric acid and histidine monohydrochloride monohydrate from British Drug Houses, Ltd., Poole, Dorset; α-methylDOPA from Sigma Chemical Company, St. Louis, Missouri; 2,4,6-trinitrobenzaldehyde from Aldrich Chemical Company, Milwaukee, Wisconsin, and bovine plasma albumin powder (Fraction V) from Armour Pharmaceutical Company, Eastbourne, Sussex.

Indomethacin [1(-*p*-chlorobenzoyl)-5-methoxy-2-methyl-indolyl-3-acetic acid] and several of its analogues were kindly donated by Drs. T. Shen and L. Sarett, Merck, Sharp and Dohme, Inc., Rahway, New Jersey; Phenylbutazone and its analogues by Dr. P. Fowler, Geigy Pharmaceutical Co. Ltd., Manchester and Dr. R. Pfister, J. R. Geigy S.A. Basel; Flufenamic and Mefenamic acids (N-α,α,α-trifluoro-*m*-tolyl- and N-2,3-xylyl-anthranlic acids) and CI-583. (N-(2,6-dichloro-*m*-tolyl)anthranilic acid by Dr. J. L. Gorringe, Parke, Davis and Co. Ltd., Hounslow. Ibufenac (4-isobutylphenylacetic acid) was supplied by Boots Pure Drug Co. Ltd., Nottingham.

METHODS

Preparation of enzymes

Substrate-specific histidine decarboxylases were extracted from the following tissues.

* Abbreviations used: Pyridoxal-5-Phosphate: PXALP; 2,4,6-Trinitrobenzaldehyde: TNBAL; Dimethylformamide: DMF; 3,4-Dihydroxy-phenylalanine: DOPA.

a. *Rat gastric mucosa.*[19] Assays were performed on the 35,000 *g* supernatant fraction from a 1:2 w/v homogenate (all glass homogenizer) of rat gastric mucosa in ice-cold distilled water.

The crude supernatant was used directly for experiments in which the radioactive assay for histamine formation was employed (see below). Otherwise the supernatant was first eluted from a Sephadex G-25 column to remove pre-formed histamine, which would otherwise interfere with the fluorimetric measurement of histamine formed during the experiment.

b. *Rat foetal tissue.*[20] Substrate-specific histidine decarboxylase activity was assayed in a protein solution obtained from the supernatant fraction of a 1:2 w/v homogenate (Waring Blendor) of whole foetuses (day 18–20) in 0·1 M sodium acetate pH 4·5. The supernatant fluid was fractionated using solid ammonium sulphate when the active protein was precipitated between 25 and 45 per cent saturation. This fraction was dissolved in 0·1 M sodium phosphate, pH 6·8 and then refractionated using saturated ammonium sulphate solution. The 25–45 per cent fraction was redissolved in the same buffer and either dialysed against it overnight at 2° or eluted from a Sephadex G-25 column made up in this buffer. This desalting procedure was essential in order to remove ammonium ions which would otherwise interfere with the assay method. The enzyme preparation was stable when stored at −75°. A 50 per cent loss in activity occurred within 3 days when this preparation was stored at −15°.

Estimation of histamine

Two methods were employed to estimate histamine, newly formed from histidine.

1. ^{14}C-Histamine formation was determined after paper chromatography by a method very similar to that described by Somerville.[21] This procedure was used to measure histamine formation by the enzyme from gastric mucosa. Incubation mixtures were made up as follows:

0·1 ml ^{14}C-histidine (0·5 μc, 0·14 mM) in 0·01 N HCl;
0·1 ml non-radioactive substrate (4·8 mM) in 0·1 M sodium phosphate, pH 6·8;
0·1 ml drug in 0·1 M sodium phosphate, pH 6·8;
0·1 ml 0·1 M sodium phosphate, pH 6·8;
0·2 ml enzyme solution, containing PXALP.

1·9 ml of the enzyme solution were pre-incubated at 37° with 0·1 ml 0·8 mM PXALP in 0·1 M sodium phosphate, pH 6·8 for 10 min. This solution was added last to the incubation mixtures. Ten microlitres of this mixture was immediately withdrawn for analysis and chromatographed on Whatman 3 MM paper by the method of Some ville (using isopropanol; 0·88 ammonia; water 100:5:10 by volume). Ten-microlitre samples of the reaction mixture were withdrawn at intervals during the incubation and chromatographed in the same way. The portions of the paper corresponding to substrate and product were assayed for radioactivity by scintillation counting in 7 ml of toluene phosphor (containing 4 g PPO and 0·1 g dimethyl POPOP/l). As controls, parallel incubations were carried out without drugs and also with heat-denatured (boiled) enzyme. All drugs were screened for possible effects on the separation of histamine from histidine, by running control chromatograms of a known mixture of the amino acid and the amine in the presence and absence of drugs.

2. Alternatively, histamine formation was measured fluorimetrically. This method was used with both enzyme preparations and is based on the methods described by

Burkhalter[22] and Håkanson.[23] Incubation mixtures contained varying amounts of all components with the exception of aminoguanidine, which was always present at 0·1 mM. All components of the incubation mixture were dissolved in 0·1 M sodium phosphate, pH 6·8 and the final volume was 3·0 ml. Drugs and PXALP were pre-incubated with the enzyme for 10 min at 37° before adding substrate. Heat-killed and drug-free controls were simultaneously incubated. The reaction was terminated by adding 0·3 ml of 6 M perchloric acid followed by heating to 100° for 2 min. The protein precipitate was removed by centrifugation and the supernatant transferred to a large stoppered tube containing 1 g sodium chloride and 0·5 ml 10 N sodium hydroxide: 15 ml of a 3:2 v/v mixture of *n*-butanol and chloroform was added and the mixture shaken mechanically for 5 min. After separation of the layers by centrifugation the organic (upper) layer was removed and washed with 5 ml 0·1 N sodium hydroxide saturated with sodium chloride; then 10 ml of this organic layer was mixed with 5 ml *n*-heptane and 5 ml 0·1 N hydrochloric acid and shaken for 5 min. The organic phase was then removed by suction and 2 ml of the acid layer assayed for histamine by the method of Shore *et al.*[24] Standard amounts (0·1–1·0 μg) of histamine were put through the same extraction procedure. The recovery of histamine was consistently in the range 62–64 per cent. A further 2 ml fraction was assayed for fluorescence due to compounds other than histamine, by reversing the order in which the developing agents were added. This residual fluorescence was subtracted from the histamine value to give a true measure of histamine formation.

Binding of PXALP *to bovine plasma albumin*[25]

Two millilitres of 0·3 mM bovine plasma albumin in 0·1 M sodium phosphate pH 7·5, was mixed with 2 ml of 0·3 mM PXALP in the same buffer and with 2 ml 3·0 mM drug in this buffer or (in controls) with 2 ml of buffer alone. After 90 min at room temperature, the absorption spectrum between 300 and 500 mμ of the mixture was recorded on an Optica CF4R recording spectrophotometer as a difference spectrum against a reference cell containing 0·1 mM plasma albumin and 1·0 mM drug (absent in controls) in 0·1 M sodium phosphate, pH 7·5. The degree of inhibition of PXALP binding was measured mainly by the percentage shift of the absorption peak of PXALP-albumin at 410 mμ towards the absorption peak of free PXALP at 387 mμ. In some cases where absorption by the drug was weak at this wavelength, it was possible to use the magnitude of the PXALP-albumin peak at 332 mμ relative to drug-free controls and other controls without albumin and drugs (i.e. free PXALP), as a measure of the degree of inhibition.

Binding of 2,4,6-*trinitrobenzaldehyde* (TNBAL) *to bovine plasma albumin*

Two millilitres of 0·3 mM plasma albumin was mixed with 2 ml of 0·3 mM TNBAL and 2 ml of 3·0 mM drug, all in solution in 0·1 M sodium phosphate pH 7·5. Drug-free controls contained 2 ml buffer (not drug solution). After 30 min at room temperature, the absorption spectrum of the solution was measured, as a difference spectrum, over the range 350–550 mμ. The degree of inhibition of TNBAL binding was calculated as the percentage decrease in the light absorption at the maxima in the regions 425–435 mμ (usually read at 425 mμ) and 525–535 mμ (usually read at 526 mμ), relative to drug-free controls. Less soluble drugs were added in N,N-dimethylformamide (DMF) solution to give a final DMF concentration of 3% v/v.

Binding of drugs to bovine plasma albumin

This was measured by equilibrium dialysis. Five millilitres of protein solution (0·1 mM plasma albumin in 0·1 M sodium phosphate, pH 6·8) was dialysed against 10 ml of drug solution (1·0 mM in the same buffer) for 24 hr, at room temperature (16–18°). Control experiments with 5 ml of buffer replacing the protein were run simultaneously. The concentration of the drug in the dialysing medium was measured spectrophotometrically, at the appropriate absorption maximum, before and after dialysis.

Chemical modification of proteins

1. Acetylation.[26] One gramme of protein was dissolved in 20 ml of 60% saturated sodium acetate solution and cooled to −2° in a freezing mixture. 1·2 millilitres of acetic anhydride was added drop by drop with continual stirring over a period of 1 hr keeping the temperature at −2°. The solution was then stirred for a further 30 min and then dialysed against changes of buffer for at least 16 hr.

2. Nitroguanidination. 1-Nitroguanidyl-3,5-dimethylpyrazole was synthesized from nitroaminoguanidine and acetylacetone.[27] The product was dissolved at pH 11, adjusted to give a 0·1 M solution at pH 10 and stirred with bovine plasma albumin for 12 hr at room temperature. After neutralization and filtration the protein solution was dialysed against changes of 0·1 M sodium phosphate, pH 7·5.

Measurement of the concentration of protein in solution

Normally the biuret method[28] was used, as this method can still be employed when the amino groups of the protein have been substituted. Bovine plasma albumin was used as a standard and protein concentrations were expressed as mg/ml. In experiments with plasma albumin (assumed to have a molecular weight of 67,000), its concentration was expressed as a millimolarity. Free (residual) amino groups were estimated by the ninhydrin method.[29]

RESULTS

Activity of the foetal enzyme preparation

The activity obtained was normally in the range 0·5–1·0 μg free base formed per mg protein/hr. The reaction was always linear over the 1st hr and often for much longer than this. The enzyme as prepared above had less than 20 per cent of the activity found in the presence of added pyridoxal phosphate, and therefore required additional cofactor for maximal activity. By varying PXALP concentration in the presence of a constant amount of substrate and performing a double reciprocal plot of 1/V:1/[PXALP], a *Km* of $2{\cdot}7 \times 10^{-7}$ M for the cofactor (allowing for residual bound PXALP), at pH 6·8, 37° and 1·0 mM histidine, was obtained. Similarly *Km* for histidine at pH 6·8, 37° and $5{\cdot}0 \times 10^{-7}$ M PXALP, was 4×10^{-4} M. The enzyme was insensitive to α-methylDOPA at a concentration of 1×10^{-4} M.

Effect of selected drugs on the foetal enzyme preparation (*Tables* 1 *and* 2). All the acidic anti-inflammatory drugs examined by us inhibited histamine formation to some degree. From these tables it can be seen that the drugs increase in potency as inhibitors in the sequence: salicylate < Ibufenac < phenylbutazone < Indomethacin < Flufenamic acid. All these drugs are non-competitive inhibitors with respect to histidine

and competitive with respect to PXALP. However, none of the anti-inflammatory drugs tested, with the possible exception of flufenamic acid, were as potent as two MAO inhibitors, NSD 1034 (82 per cent inhibition at 10^{-5} M) and NSD 1055 (100 per cent inhibition at 10^{-5} M).

TABLE 1. RELATIVE POTENCIES OF SOME ANTI-INFLAMMATORY DRUGS IN INHIBITING FOETAL HISTIDINE DECARBOXYLASE ACTIVITY (SUBSTRATE CONCENTRATION VARIED)

Drug	Apparent *Ki* (mM)
Sodium salicylate	2·7
Ibufenac	2·0
Phenylbutazone	0·7
Indomethacin	0·4
Flufenamic acid	0·05

Inhibition relative to histidine was non-competitive for above details.

Incubations contained 2·0, 4·0 and 8·0 × 10^{-3} M histidine, 5 × 10^{-7} M PXALP, 1 × 10^{-4} M aminoguanidine hydrogen carbonate and varying concentrations of drugs in 3·0 ml of phosphate buffer pH 6·8 and usually 2·0 mg protein. Time of incubation, 1 hr at 37°. Apparent *Ki* and type of inhibition were obtained graphically by Dixon's method[30] from the point of intersection on the inhibitor concentration axis.

TABLE 2. RELATIVE POTENCIES OF SOME ANTI-INFLAMMATORY DRUGS IN INHIBITING FOETAL HISTIDINE DECARBOXYLASE ACTIVITY (COENZYME CONCENTRATION VARIED)

Drug	*Ki* (mM)
Sodium salicylate	1·2
Ibufenac	0·7
Phenylbutazone	0·4
Indomethacin	0·2
Flufenamic acid	0·03

Inhibition relative to PXALP was competitive throughout.

Incubations contained 1·0 × 10^{-3} M histidine, 1·0, 2·5 and 5·0 × 10^{-7} M PXALP, 1·0 × 10^{-4} M aminoguanidine hydrogen carbonate and varying concentrations of drugs in 3·0 ml of phosphate buffer pH 6·8 and usually 2·0 mg protein. Time of incubation 1 hr at 37°. *Ki* and type of inhibition were obtained graphically by Dixon's method.[30]

Activity of the gastric enzyme preparation

The activity normally obtained was in the range 0·1–0·2 μg free base formed per mg protein/hr. As was shown by Kahlson[31] and by Castellucci[32] the activity of the enzyme increased from a minimum in the starved animal to a maximum 3–5 hr after

feeding. In these experiments the animals were killed within 4 hr of refeeding following overnight starvation. Filtering the protein through a Sephadex G-25 column resolved the cofactor from the apoenzyme; adding PXALP restored maximal activity to the extract. Under these conditions the enzymic activity was linear for at least 1 hr and often for longer. *Km* for histidine was found to be $4{\cdot}5 \times 10^{-4}$ M at pH 6·8 and 37° with 5×10^{-7} M PXALP; *Km* for PXALP was found to be 5×10^{-7} M at pH 6·8, 37° with 10^{-3} M histidine. The enzyme was not inhibited by 10^{-4} M α-methyl-DOPA.

These results were obtained using the fluorimetric method of estimating histamine. This method was found to be far more reliable than the radioactive method, which was developed only as a possible technique for the rapid screening of drugs.

The effect of anti-inflammatory drugs and their chemical analogues on histamine formation in vitro *by the gastric enzyme*

From Table 3 it can be seen that there is reasonable correlation between the results

TABLE 3. THE INHIBITION OF HISTIDINE DECARBOXYLASE ACTIVITY IN A GASTRIC MUCOSAL PREPARATION BY SOME ANTI-INFLAMMATORY DRUGS AND THEIR CHEMICAL ANALOGUES

Drug added	Relative drug activity			
	Radiochemical assay*		Fluorimetric assay†	
	Concn. mM	% Inhibition	Concn. mM	% Inhibition
Sodium salicylate	1·0	30	2·0	27
Sodium 4-hydroxybenzoate	1·0	17	2·0	11
Sodium gentisate	1·0	80	2·0	36
Sodium γ-resorcylate	1·0	70	2·0	45
Sodium benzoate	1·0	6	2·0	7
Acetylsalicylic acid	1·0	17	2·0	20
Salicylamide			2·0	37
Hexahydrosalicylic acid (*cis*)			2·0	11
Phenylbutazone	0·5	43	1·0	42
Oxyphenbutazone	0·5	20	1·0	32
Sulphinpyrazone	0·5	51	1·0	48
Amidopyrine	0·5	5	1·0	0
Phenazone			1·0	0
4-Hydroxyphenazone	0·5	18	1·0	15
Rubazonic acid			0·25‡	0
Mefenamic acid	0·5	40	1·0	49
Flufenamic acid	0·5	52	1·0	83
CI 583			0·5‡	46
N,Methylanthranilic acid	0·5	0	1·0	0
Cinchophene	0·5	60	0·5	34
3-Hydroxycinchophene	0·5	76	0·5	48
8-Hydroxycinchophene			0·5	44
Ibufenac			2·0	34
Phenylacetic acid			2·0	19
Lauric acid			2·0	58
Perfluoro-octanoic acid			0·5	41
(5-Methoxy)indomethacin	0·5	65	0·5	32
5-Fluoro analogue of indomethacin	0·5	68	0·5	50
5-Methoxyindole-3-acetic acid	0·5	0	0·5	0

TABLE 3—*continued.*

Drug added	Relative drug activity			
	Radiochemical assay*		Fluorimetric assay†	
	Concn. mM	% Inhibition	Concn. mM	% Inhibition
Sodium aurothiomalate			1·0	16
Sodium aurothiosulphate			1·0	70
Sodium thiosulphate			1·0	0
SNR 1804			1·0	44
Pentachlorophenol			0·5	77
Biogastrone			1·0	85

* Radioactive assay. Incubation volume 0·6 ml. Final concentration of reactants, histidine $8{\cdot}2 \times 10^{-4}$ M, PXALP $1{\cdot}35 \times 10^{-5}$ M. Incubation time 1 hr, at pH 6·8 and 37°.

† Fluorimetric assay. Incubation volume 3·0 ml. Final concentration of reactants, histidine 1×10^{-3} M, PXALP 5×10^{-7} M. Incubation time 1 hr at pH 6·8 and 37°.

‡ Concentration of drug limited by solubility.

obtained by the fluorimetric and the radiochemical methods for determining newly-formed histamine in the presence of these drugs, but that the radiochemical method consistently indicated a higher degree of inhibition for any given level of an active compound. In order to use the fluorimetric method for determining histamine, it was necessary first to remove the considerable amount of histamine present in the crude enzyme preparation by passing the preparation through a Sephadex G-25 column. This treatment also removed the PXALP cofactor from the protein.

TABLE 4. RELATIVE BINDING OF SOME DRUGS TO BOVINE PLASMA ALBUMIN AT pH 6·8

Drug	μ moles bound/μ mole protein
Sodium salicylate	2·5
Phenylbutazone	4·0
Indomethacin	5·5
Flufenamic acid	7·1

Dialysis for 24 hr at 18 ± 2° in the presence of 0·1 M sodium phosphate, pH 6·8. 10 μ moles drug outside the sac, 0·5 μ moles protein inside the sac.

Because the enzymic activity of this fraction was very low, the recovery of radioactive histamine was not much above the count obtained with "killed" enzyme controls nor was it very much greater than the background count of the scintillation counter. Hence the usefulness of this radiometric method for screening drugs for inhibitory activity is limited. Considerably more reliance can be placed on the results obtained by the fluorimetric method and the greater accuracy of this method outweighs the fact that it is more cumbersome.

It is noticeable that the gastric enzyme seems to be less sensitive to drugs than does the foetal enzyme. However the gastric enzyme preparations contained more protein than the foetal enzyme preparations and this "extra" protein, if it binds the drugs, will reduce the concentration of drug available to inhibit the histidine decarboxylase.

The binding of aldehydes to plasma albumin

1. PXALP. The interaction of equimolar amounts of plasma albumin and PXALP has been well documented by Dempsey and Christensen,[25] who showed that at pH 7·5, two complexes were formed. The first complex had an absorption maximum in the region 410–415 mμ and reacted further to form the second complex, which had an absorption maximum at 332 mμ. They showed that certain anions with structures rather similar to that of PXALP could inhibit the formation of these complexes (Fig. 1). The results in Table 5 show that this property of inhibiting (and reversing)

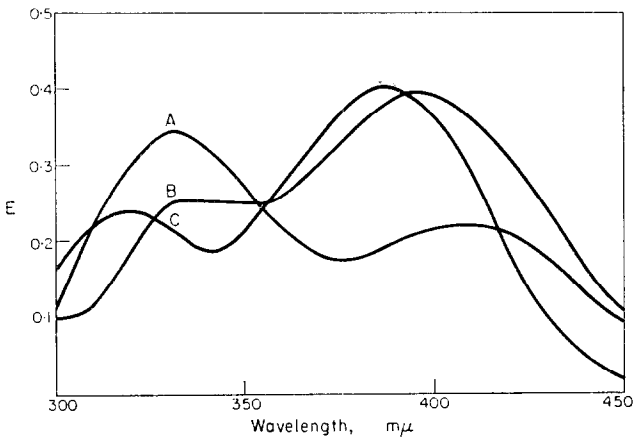

FIG. 1. Absorption spectra of the PXALP-Albumin complexes.
A. 0·1 mM albumin, 0·1 mM PXALP in 0·1 M sodium phosphate pH 7·5, ionic strength 0·27, 18°, 90 min after mixing.
B. The same in the presence of 1·0 mM Phenylbutazone (corrected for drug absorption).
C. Free PXALP 0·1 mM.

TABLE 5. THE EFFECT OF ANTI-INFLAMMATORY DRUGS AND SOME OF THEIR CHEMICAL ANALOGUES ON THE BINDING OF PYRIDOXAL PHOSPHATE AND 2,4,6-TRINITROBENZALDEHYDE TO BOVINE PLASMA ALBUMIN

Drug added	Concn. (mM)	% Inhibition withPXALP % shift 410→ 387 mμ	% Inhibition with TNBAL at 425 mμ	at 526 mμ
Sodium salicylate	1·0	72	31	31
Sodium 4-hydroxybenzoate	1·0	55	22	20
Sodium gentisate	1·0	61	31	27
Sodium γ-resorcylate	1·0	83	41	42
Sodium benzoate	1·0	55	0	3
Acetylsalicylic acid	1·0	61	9	9
Salicylamide	1·0		0	0
Hexahydrosalicylic acid	1·0		0	0
Phenylbutazone	1·0	72	45	37
Oxyphenbutazone	1·0	50	50	50
Sulphinpyrazone	1·0	61	4	4
Phenazone	1·0	0	5	5
	1·0	0	0	0

TABLE 5—*continued.*

Drug added	Concn. (mM)	% Inhibition with PXALP % shift 410→ 387 mμ	% Inhibition with TNBAL at 425 mμ	at 526 mμ
1-Phenyl-2-methyl-3-carboxy-4-dimethylamino-pyrazol-5-one	0·5	0	0	0
Rubazonic acid	0·25*		26	—
N-Methylrubazonic acid	0·25*		0	0
Mefenamic acid	1·0	50	57	55
Flufenamic acid	0·20		27	27
	0·50		50	50
	1·0	70	74	73
CI 583	0·5*		57	62
N-Methylanthranilic acid	1·0	50	35	30
Anthranilic acid	1·0		9	6
Phenylacetic acid	1·0		0	0
Ibufenac	1·0		47	47
Lauric acid	1·0		76	75
Perfluoro-octanoic acid	1·0		60	60
(5-Methoxy)indomethacin	0·2		23	25
	0·5		61	60
	1·0	100	75	81
(5-Fluoro)indomethacin	1·0	90	70	76
Indomethacin amide	*	0	0	0
5-Methoxyindoleacetic acid	1·0	10	23	23
Cinchophene	1·0	61	54	55
3-Hydroxycinchophene	1·0		75	83
8-Hydroxycinchophene	1·0		68	74
Isonicotinic acid	1·0		5	8
Acridine-9-carboxylic acid	1·0		40	38
Sodium aurothiomalate	1·0		9	4
Sodium aurothiosulphate	1·0		40	40
cis-p-Coumaric acid	1·0		15	14
trans-p-Coumaric acid	1·0		21	18
Chloroquine phosphate	1·0		0	0
Chloroquine sulphate	1·0		0	0
Hydrocortisone	1·0		10	14
Hydrocortisone hemisuccinate	1·0		16	22
Hydrocortisone-21-aldehyde	1·0		19	26
18β-Glycyrrhetic acid	1·0		9	13
Biogastrone	1·0		54	56
2-Phenylindan-1,3-dione	1·0		49	46
SNR 1804	1·0		78	83
Pentachlorophenol	1·0		84	84

0·67% Albumin (0·1 mM), 0·1 mM aldehyde, drugs (usually 1·0 mM) all in 0·1 M sodium phosphate pH 7·5. Inhibition of pyridoxal phosphate (PXALP) binding given as percentage decrease in light absorption at 332 mμ (drug-free controls as 0 per cent and unbound PXALP as 100 per cent) and also by the percentage shift of the absorption maximum of bound PXALP (at approximately 410 mμ) towards the absorption maximum of free PXALP (at 387 mμ). Inhibition of trinitrobenzaldehyde (TNBAL) binding given as the percentage decrease in light absorption at 425 mμ and 526 mμ due to bound TNBAL. All values were corrected for light absorption by plasma albumin. Where necessary, drugs were added in solution in N,N-dimethylformamide (DMF) giving a final DMF concentration of 3% v/v (also in controls).

* Concentration limited by drug solubility.

the reaction of PXALP with albumen is shared by a wide range of acidic anti-inflammatory drugs, and that clinically inactive chemical analogues of these drugs do not have this property, even though some of these analogues are also acids. Pre-incubation of the albumen with trypsin or α-chymotrypsin (1 mg in 2·5 ml of 2% albumen at pH 7·5) at 18° or 37° increases the rate at which these complexes are formed.

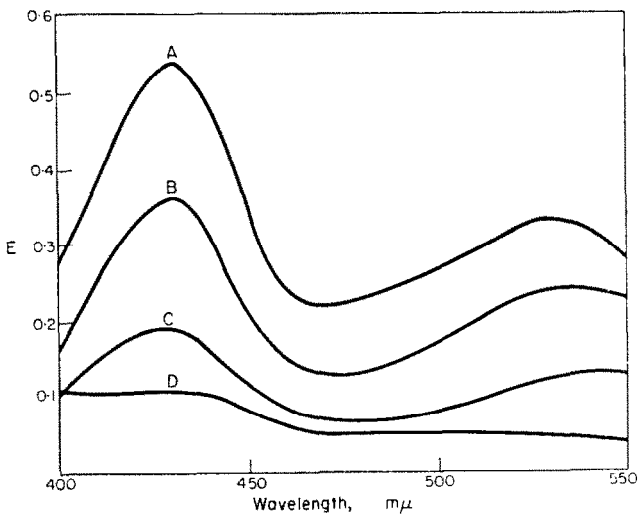

FIG. 2. Absorption spectra of the TNBAL-albumin complex(es).
A. 0·1 mM albumin, 0·1 mM TNBAL in 0·1 M sodium phosphate pH 7·5, ionic strength 0·27, 18°, 30 min after mixing.
B. In the presence of 1·0 mM sodium salicylate.
C. 1·0 mM Phenylbutazone.
D. 1·0 mM Indomethacin.

2. TNBAL. Because the absorption of PXALP-albumin at 332 mμ is often masked by absorption by drugs at the same wave-length, a further model system using the reaction between TNBAL and albumin (in the same proportions and at the same pH) has been developed. This reaction yields a product or products with absorption maxima in the range 425–435 and 525–535 mμ respectively. The results in Table 5 show that this rubigenic reaction (measured in either wave-length range) is inhibited by clinically active anti-inflammatory acids but is not significantly inhibited by their inactive analogues or by non-acidic drugs.

This reaction between TNBAL and albumin proceeds as shown in Fig. 3. The initial increase in optical density is very rapid but this slows until, about 10 min after mixing, the optical density is increasing at a uniform rate which is maintained for about a further 20 min, after which the rate of increase in optical density slows still further. The effect of drugs is to slow the initial rate and, in the case of the more potent drugs, to lower the rate of the second stage of the reaction as well. The values quoted in Table 5 give a measure of the extent to which the reaction has been inhibited 30 min after mixing rather than the extent to which either stage of the reaction is inhibited.

The rate of the reaction was decreased by increasing the ionic strength (Fig. 4) However, it was also found that the rate of the drug-inhibited reaction was lowered with increasing ionic strength to the same extent as was the drug-free reaction. Increasing concentrations of a denaturing agent, urea, also decreased the rate of the reaction.

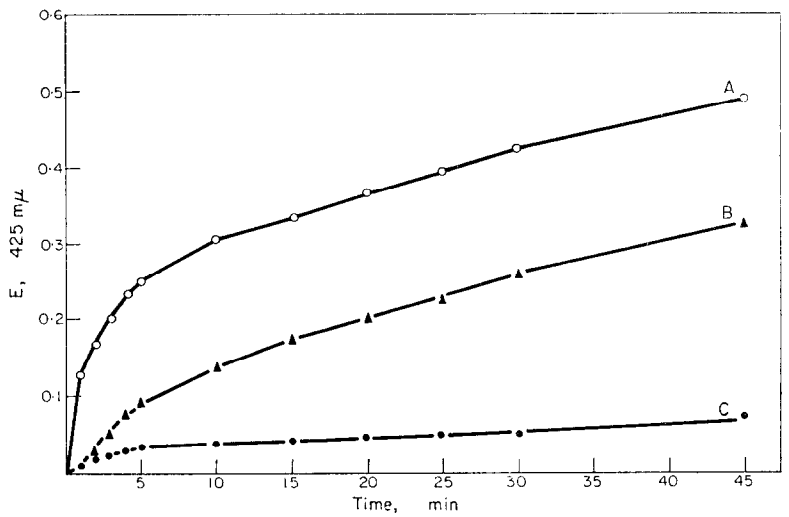

FIG. 3. Increase in extinction (1·0 cm cell) at 425 mμ with time after mixing TNBAL and albumin.
A. 0·1 mM TNBAL, 0·1 mM albumin in 0·1 M sodium phosphate pH 7·5, ionic strength 0·27, 18°.
B. In the presence of 1·0 Mm sodium salicylate.
C. In the presence of 1·0 mM Indomethacin.

If the N-terminal α-amino group and the many ε-amino groups of the lysyl residues of the albumin are modified either by acetylation or by nitroguanidination so that at least 93 per cent of the ninhydrin-reacting groups become unreactive, the reaction of TNBAL with the protein is inhibited, but not completely (Fig. 5). Pre-incubation of native albumin with trypsin or α-chymotrypsin (1·0 mg in 2·5 ml of 2% albumin, pH 7·5) at 18° and 37° diminished the rate of reaction between the (digested) protein and TNBAL (contrast with the reaction between PXALP and digested albumin).

The following substances failed to react in the same way as albumin with TNBAL: Lysine, lysine methyl ester, 6-aminohexoic acid, 6-aminohexoic methyl ester, imidazole, imidazoleacetic acid and histidine (all at 33·3 mM).

The following compounds reacted with TNBAL in a manner similar to acetylated albumin: arginine, arginine methyl ester, N-(α)-benzoylarginine methyl ester (all at 33·3 mM) and salmine sulphate (33·3 mM in arginine side chains, 0·1 mM in lysine side chains).

Poly-L-lysine reacted in the same way as albumin initially but there was no secondary reaction. Calf thymus histone (mol. wt. 42,000) at a concentration of 0·1 mM (4·5 mM in lysine, 2·5 mM in arginine) reacted in a similar manner to, but not as rapidly as, albumin.

DISCUSSION

Concerning the enzymes

The activity of both foetal and gastric histidine decarboxylases is very low, often only sub-microgram quantities of histamine being formed in the course of the reaction. These two enzymes are peculiar in that their cofactor can be separated from the

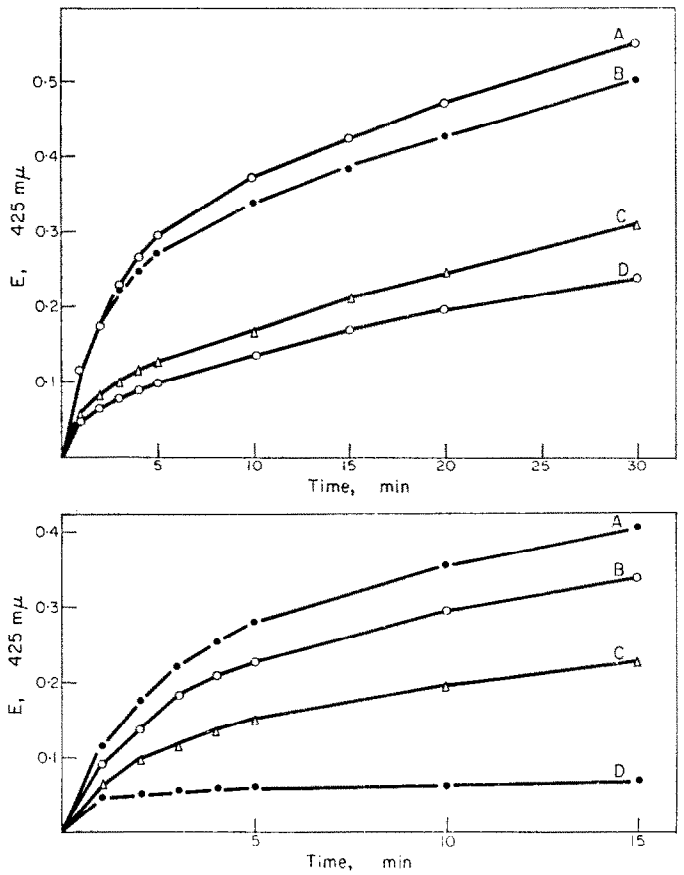

FIG. 4. The effect of ionic strength and urea concentration on the reaction of TNBAL with bovine plasma albumin.

Upper graph: Effect of ionic strength. 0·1 mM albumin, 0·1 mM TNBAL, pH 7·5, 18°.
A. $I = 0{\cdot}108$; B. $I = 0{\cdot}270$; C. $I = 0{\cdot}400$; D. $I = 0{\cdot}500$.

Lower graph: Effect of urea concentration, 0·1 mM albumin, 0·1 mM TNBAL, pH 7·5, 0·1 M sodium phosphate, 18°.
A. 0·00 M urea; B. 0·667 M urea; C. 2·667 M urea; D. 5·33 M urea.

apoenzymes by gel-filtration or by dialysis.* This is also a property of the "inducible" histidine decarboxylase described by Schayer which plays a key role in his theory of microcirculatory control and inflammation.[33] While it is very unlikely that the two enzymes which we have studied would normally play a direct role in the inflammatory

* It has now been reported that a partially purified preparation of histidine decarboxylase from rat stomach has properties very similar to those of histidine decarboxylase from rat foetal tissue.[53]

response, their properties (including drug sensitivity) serve as a reasonable guide to the behaviour of the "inducible" enzyme in the presence of anti-inflammatory drugs.

Furthermore it seems at present that all clinically active anti-inflammatory drugs affect the gastric mucosa to some extent, some causing overt gastric erosion. There may possibly be some causal connection between this phenomenon and the inhibition of gastric histidine decarboxylase by these drugs.

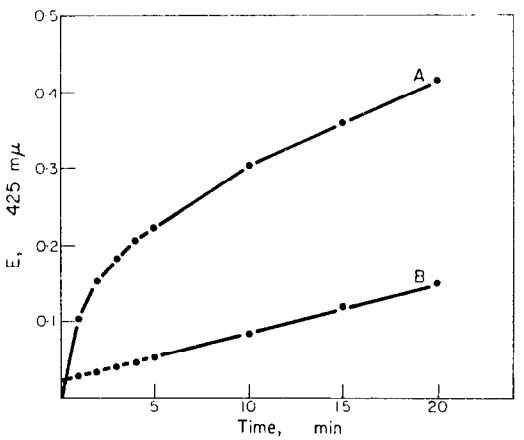

FIG. 5. Reaction of native and nitroguanidinated albumin with TNBAL.
A. 0·1 mM native protein, 0·1 mM TNBAL in 0·1 M sodium phosphate pH 7·5, ionic strength 0·27,18°.
B. 0·1 mM nitroguanidinated protein (95 per cent amino groups substituted) under the same conditions.

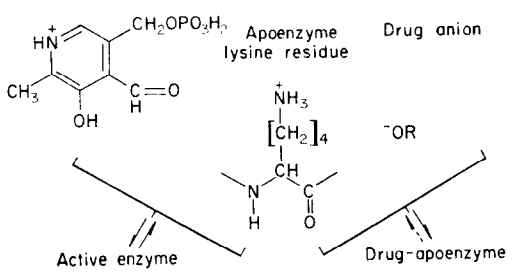

FIG. 6. Proposed mechanism for the inhibition of histidine decarboxylase by acidic drugs.

The mechanism of drug action on histamine formation in vitro

Our experimental results support the conclusion that the mechanism of drug action is as outlined in Fig. 6, that is, there is competition between drug anion and PXALP for a lysine ε-amino group which is part of the active site of the enzyme. The evidence for this hypothesis is as follows:

1. All the drugs which we have shown to inhibit histamine formation *in vitro* are acidic molecules. Neutral and basic drugs have no inhibitory activity of this type.

2. These acidic drugs have been shown to compete with PXALP and TNBAL for reactive groups on plasma albumin. The changes in the aldehyde-binding properties

of albumin after chemical modification of the protein indicate that these groups are lysine ε-amino groups. Neutral and basic drugs are not able to displace these aldehydes from albumin.

3. In the case of several drugs which are known to be clinically active, their relative activity as inhibitors of histamine formation *in vitro* is paralleled not only by their relative activity as inhibitors of aldehyde-binding to albumin but also by the extent to which individual drugs themselves bind to albumin (see results section). This drug binding is also inhibited by modifying the albumin ε-amino groups.

4. The cofactor PXALP is easily dissociated from the enzymes with which we have worked, though not from other decarboxylases.

5. In the case of the five clinically active drugs which we have studied in detail, the inhibition of histamine formation has been shown to be non-competitive with respect to histidine but competitive with respect to PXALP.

The ionic interaction of the anion with the ε-amino group is not the only bonding between drug and protein determining drug activity, as several acidic analogues of the active drugs are not efficient inhibitors of histamine formation nor are they able to displace bound aldehydes from albumin. It appears that in the case of the more potent drugs there is considerable and less specific non-ionic binding to the proteins which is not abolished by chemical modification of the ε-amino groups of the protein. It may well be that this second mode of binding (hydrophobic in character?) is responsible for the fact that the more potent drugs alone are able to inhibit the second stage of the TNBAL reaction. Perhaps the secondary TNBAL reaction (which is not fully sensitive to the blocking of lysine groups) involves (further) binding of the aldehyde to another type of group or zone on the albumin, with which only the more potent drugs interact.

The trinitrobenzaldehyde–albumin reaction

It is possible to assume, by drawing parallels with the PXALP–albumin reaction, that the fundamental reaction of TNBAL with albumin involves the formation of an azomethine (Schiff base) between the carbonyl group of the aldehyde and a lysyl ε-amino group on the protein. However, the presence of a carbonyl group attached to an aromatic nucleus is not the only requirement for the reaction, as benzaldehyde, 2-nitro- and 2,4-dinitro-benzaldehyde(s) will not react with albumin to anything like the same extent. Less specific interactions also seem to play a part. The evidence for this is as follows:

1. In the superficially comparable PXALP-albumin reaction the phosphate ester group is also essential for protein binding.

2. In the case of the TNBAL-albumin reaction, the nitro groups appear to be necessary. However, because neither picric acid nor the nitrobenzoic acids react in the same way, the TNBAL reaction is not entirely due to the nitrated aromatic nucleus.

3. Increasing the ionic strength of the solution (with sodium chloride) decreases the extent of the reaction, indicating that there is some role played by ionic interactions.

4. Because a denaturating agent such as urea decreases the extent of the reaction it is probable that there is some interaction between the aromatic nucleus and the native form of the protein.

5. Partial enzymic hydrolysis of the protein to peptides of lower molecular weight decreases the extent of the reaction. Furthermore, small molecular weight proteins

rich in lysyl residues and several lysine derivatives do not react with the aldehyde. Arginine-rich proteins and arginine derivatives react with the aldehyde but in a manner analogous to poly N-(ε)-acetyl albumin. This indicates that a further interaction of the aldehyde might be with the arginine residues of the protein (this might be responsible for the second stage of the TNBAL-albumin reaction, and might explain why the second stage of the reaction is inhibited only by drugs which bind to the protein more firmly than salicylic acid).

6. Further evidence that TNBAL reacts with lysyl amino groups on proteins is afforded by the fact that this aldehyde alone (or N-substitution of ε-amino groups) inhibits tryptic digestion of lysine-rich, arginine-deficient proteins,[17] and will also under certain conditions inhibit enzymes which have a lysyl ε-amino group at their active catalytic centre, e.g. acetoacetic acid decarboxylase of *Cl. acetobutylicum* (Whitehouse and Skidmore, unpublished observations).

The use of aldehyde-binding to albumin as a screening method for potential anti-inflammatory drugs

The reaction of PXALP with plasma albumin is complex, involving at least two binding-sites on the protein when the reactants are in equimolar proportions. The resulting complex spectrum, coupled with the fact that the wave-length range at which the PXALP-protein interaction is readily observed overlaps with the absorption bands of many clinically important aromatic drugs, makes this system rather difficult to use. However, in such cases as it has been applied, the correlation between known clinical activity, inhibition of *in vitro* histamine formation and inhibition of PXALP-binding is acceptable. In the relatively few cases where the drug-albumin-TNBAL reaction appears to be abormal (e.g. with sulphinpyrazone), studying PXALP-binding in the presence of drugs can still be very useful.

The TNBAL-albumin reaction offers the advantage that the appearance of the absorption peaks and the drug effect can be seen by eye. The drug effect appears as a partial quenching (to the same extent at both wave-lengths) of the red coloration which is normally formed. Furthermore, it is usually the case that one (if not both) of these peaks is outside the absorption range of the drug being studied, permitting quantitative comparison to be made spectrophotometrically between different drugs. In most cases it has been found that there is fairly good correlation between the drug sensitivity of the PXALP-albumin and TNBAL-albumin reactions. There are some rare exceptions though, notably sulphinpyrazone; to which the TNBAL system is almost insensitive even though the PXALP-albumin reaction and histamine formation are sensitive to this particular drug.

Relationship of drug activity in vitro *to drug activity* in vivo *and to chemical structure*

Comparison of Tables 3 and 5 indicate the generally close parallel between the action of a given drug in inhibiting histamine formation and in displacing either PXALP or TNBAL from its combination with plasma albumin. The structure–activity relationship in both respects would appear to agree with the published data available regarding the relationship of anti-inflammatory activity *in vivo* to chemical structure for any one type of anti-inflammatory acid,[34–37] with the possible exception of the hydroxy-salicylates (discussed below). Non-acidic anti-inflammatory drugs

such as chloroquine, hydrocortisone and amidopyrine had no appreciable effect on either histamine formation *in vitro* or on the TNBAL-albumin reaction.

Rubazonic acid was the only acidic metabolite of amidopyrine with any notable drug activity *in vitro*, with an activity comparable to that of flufenamic acid and indomethacin in inhibiting histamine formation and the TNBAL-albumin colour reaction (and also in uncoupling oxidative phosphorylation[38]). 3,5-Dimethylpyrazole is known to be oxidized to a methylpyrazole carboxylic acid:[39] the corresponding oxidation products (hypothetical metabolites) of amidopyrine (and of phenazone), and 4-hydroxyphenazone (another acidic metabolite) were not notably effective drugs *in vitro* in these present tests for potential anti-inflammatory activity. Amidopyrine itself was inactive: this drug also fails to inhibit the heat-cogulation of plasma albumin at pH 5·3 (Y. Mizushima, personal communication)—a property of many acidic anti-inflammatory drugs.[40, 41] Furthermore, amidopyrine is devoid of uncoupling activity—another property of acidic anti-inflammatory drugs.[42] However amidopyrine is reported to antagonize histamine *in vivo*[43] and therefore its anti-inflammatory activity might be due to an anti-histamine (action) effect, whereas the acidic drugs described here are in fact also anti-histamine (biogenesis) drugs but with a mode of action distinct from that of the conventional "anti-histamines".

The greater *in vitro* potency of (i) γ-resorcylate and gentisate compared with salicylate, (ii) "Biogastrone" (the hemi-succinate of 18β-glycyrrhetic acid) compared with glycyrrhetic acid itself and (iii) the hydrocinchophenes compared with cinchophene, indicate that the introduction of a hydrophilic group into the molecule does not necessarily reduce the drug activity *in vitro* and may actually potentiate it. By contrast each of these more polar compounds, with the exception of 3-hydroxycinchophene, is actually less potent than the parent compound in uncoupling oxidative phosphorylation *in vitro*.[34] It would, therefore, be very useful to have further data on the relative *in vivo* activities of these particular compounds, both as anti-inflammatory drugs in animals and as antirheumatic agents in man, in order to resolve (i) whether uncoupling of oxidative phosphorylation[34, 42] or ability to inhibit histamine formation *in vitro* is a better experimental guide for detecting potential anti-inflammatory activity, and relative importance of lipophilic character in determining *in vivo* anti-inflammatory activity.

The results indicate that, on the basis of inhibiting histamine formation *in vitro*, flufenamic acid might be expected to be more active *in vivo* than indomethacin. Currently available clinical reports suggest that the effective daily doses for antirheumatic activity in man are about the same for both drugs (0·3 g/day flufenamic acid, 0·2 g/day indomethacin). This could be explained by the finding that flufenamic acid binds to albumin more readily than does indomethacin, with the result that the greater potential activity of flufenamic acid is masked—because the greater the degree of binding to protein, the lower the unbound (therapeutically available) circulating concentration of drug.

The coumaric acids were included in this survey because of their reported activity *in vivo*, but proved to be virtually inactive: they also fail to uncouple oxidative phosphorylation significantly. Both SNR 1804 (2(3,4-dichlorobenzamido)-phenoxyacetic acid)[44] and trifluorobenzoylacetone exhibit anti-inflammatory activity in granuloma and oedema assays *in vivo* (Smith and Nephew Research Ltd., private communication) and also uncouple oxidative phosphorylation at 0·1 mM (Whitehouse and Skidmore,

unpublished observations). At least one other dione which uncouples oxidative phosphorylation, 2-phenylindan-1,3-dione,[45] exhibits anti-inflammatory activity *in vivo*[46] and inhibits the TNBAL reaction.

Furthermore, other acidic compounds not normally regarded as anti-inflammatory drugs but which bind firmly to (the ϵ-amino groups of) plasma albumin, such as pentachlorophenol[47] and perfluoro-octanoic acid[48] also inhibit both histamine formation *in vitro* and the TNBAL reaction.

Drug activity of this type is not restricted to organic compounds as is shown by the behaviour of sodium aurothiosulphate (Sanochrysin) a time-honoured antirheumatic drug which is certainly more potent than salicylic acid in inhibiting histamine formation and aldehyde binding (and also in uncoupling oxidative phosphorylation[49]).

Further comment

As these experiments have shown, acidic anti-inflammatory drugs and certain gold preparations (e.g. Sanochrysin) are able to displace both PXALP and TNBAL from binding sites (amino groups) on plasma albumin. A further important property of these drugs may thus be to displace other (anti-inflammatory) agents already bound to circulating albumin, such as long chain fatty acids or cortical hormones,[50] or to interfere with the serum binding of other drugs and hormones.[51] This action of the anti-inflammatory acids on the *apoenzyme* is to be contrasted with the action of other (basic) drugs which inhibit histidine decarboxylase(s) (e.g. NSD 1034 and NSD 1055)[52] by chemical combination with the *cofactor* (and whose action is presumably less readily reversed).

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