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Inhibition of enzymes by alkylsalicylic acids

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5-n-Alkylsalicylates inhibited a variety of enzymes that transform acidic substances, viz. glucose-6-phosphate dehydrogenase, glyoxalase, xanthine oxidase, carbonic anhydrase and D-amino-acid oxidase. Inhibitory potency rose to a peak at the n-nonyl derivative. None of the tests were of value, either singly or in combination, as screening processes for anti-inflammatory activity. The comparable results with a trinitrobenzaldehyde reagent suggest that the various inhibitions arise by non-specific association of the drugs with arginine and lysine residues in proteins.

Acidic anti-inflammatory agents at 10^{-3}M concentration will partially inhibit several enzymes *in vitro*. Skidmore & Whitehouse (1965) considered that inhibition of, for example, histidine decarboxylase, could arise by drug molecules associating with free amino-groups in the enzymes, so reducing their availability to pyridoxal co-enzyme. On this basis they devised a simple chemical test that attempted to relate anti-inflammatory activity to the degree of inhibition of a reddening when 2,4,6-trinitrobenzaldehyde reacted, presumably, with lysyl amino-groups in serum albumen. The test gave too many false positives to be of value for even rough screening purposes (cf. Phillips, Sancilio & Kurchacova, 1967).

Since the test gave positive results with well-known anti-inflammatory compounds, an attempt was made to eliminate at least some of the false positives by supplementary tests *in vitro*. Chemical tests will rarely be a measure of biological activity within a group of substances since they are usually a measure for a limited group of atoms within a molecule, whereas, biological activity is quantitatively dependent on the whole structure of the drug molecule. Nevertheless, a sequence of tests for different partial chemical structures within a molecule could give a degree of qualitative specificity by elimination. It was found that a combination of the trinitrobenzaldehyde test with enzymic inhibitory tests, notably that of glyoxalase, did not fulfil the expectation. This is illustrated by results with a homologous series of 5-n-alkylsalicylates in which there was a parallel between inhibitory potency in various tests with alkyl chain length. The compounds had no anti-inflammatory effect in animals.

EXPERIMENTAL

Methods

Glucose-6-phosphate dehydrogenase of rat adrenal was assayed in a Thunberg tube using an azine dye as H-acceptor (Doxiadis, Fessas & Valaes, 1961) or manometrically with ferricyanide as H-acceptor (Quastel & Wheatley, 1938). Other enzymes were prepared and assayed as described in the following references: glyoxalase of rabbit kidney (Platt & Schroeder, 1934), xanthine oxidase of milk (Dhungat & Sreenivasan, 1954), carbonic anhydrase of rat erythrocytes (Roughton & Booth, 1946), D-amino-acid oxidase of pig kidney (Bartlett, 1948), glutamic decarboxylase of guinea-pig brain (Roberts & Frankel, 1951) and succinic dehydrogenase of rat liver (Bernath & Singer, 1962).

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RESULTS AND DISCUSSION

The 2,4,6-trinitrobenzaldehyde (TNB) colour reaction with serum albumen. TNB, colourless when pure, soon turns red. It gives red products (maxima at 425, 525 nm) with various compounds but the chemistry can be obscure and complex. The products from TNB and albumen are unlikely to be simple aldimines. In a broad superficial survey of the reaction, none of a variety of amino-acids and their derivatives and polymers exceeded the tinctorial capacity of bovine serum albumen. There was large variation in albumens from different sources (Table 1). Egg albumen gave no

Table 1. *Chromogenicity of serum albumens with 2,4,6-trinitrobenzaldehyde.* Albumen (16 mg = 0·24 μmol bovine albumen), TNB (0·3 μmol in dimethylformamide), 0·1M sodium phosphate pH 7·5 (3 ml) at room temperature in the dark for 2 h. *E* taken at 425 nm. Bovine albumen = 100 (*E* =0·358–0·404). TNB control, *E*, 0·006.

Species	Colour intensity (% bovine)
Rabbit ..	74
Human	20
Guinea-pig	79
Sheep ..	109
Horse ..	32

colouration. The following comments are based on a large number of simple experiments, that need no detailed description. The red colour with albumen is probably due to a compound rather than a polynitro-complex. Absorption spectra in the visible range varied very little for the products of different reactants. No stoichiometric relation could be devised even for simple chemical reactants. (The test described in the literature specifies 1 mol TNB per mol albumen though the latter contains about 60 lysyl residues.) Contrary to hypothesis, TNB reacted more readily with guanidine (arginine) compounds than with lysine or its derivatives. Nevertheless, thermal polylysines and polyarginines had equivalent tinctorial capacity when prepared under similar conditions, the colour obtained being greater as the degree of polymerization increased. Clupeine gave a red colour. Conversion of lysyl amino-groups in egg white lysozyme to either guanidine or acetamidine (McCoubrey & Smith, 1966) had no effect on the chromogenicity of lysozyme. Other proteins, including various blood fractions, gave but weak colour development. Destruction of the guanidine groups of bovine serum albumen by malondialdehyde (King, 1966) or cyclohexan-1,2-dione (Toi, Byrum & others, 1967) virtually abolished colour formation and also the ability of albumen to associate with phenylbutazone in equilibrium dialysis experiments. Indomethacin would not inhibit colour development in the test by more than 60% at concentration up to 10^{-3} M.

The above results made it difficult to place the TNB test on a quantitative basis and there was no evidence to support the hypothesis that TNB reacted preferentially with lysyl amino-groups as opposed to arginyl residues.

Inhibition of glucose-6-phosphate dehydrogenase by anti-inflammatory agents. 5-n-Alkylsalicylates, among other substances, inhibited rat adrenal glucose-6-phosphate dehydrogenase when tested in a Thunberg tube assay using brilliant cresyl

blue as the final H-acceptor, and either glucose-6-phosphate or 6-phosphogluconate as substrate. The peak inhibitory activity was at the n-decyl derivative, (Table 2).

Table 2. *Inhibition of enzymes by 5-n-alkylsalicylates.* Drugs are at 10^{-3}M unless shown otherwise. Glucose-6-phosphate dehydrogenase assay with brilliant cresyl blue (Thunberg) or ferricyanide (manometric) as H-acceptor. Values for the Thunberg method are ratios of test to control times for dye bleaching. Values elsewhere are % inhibition. TNB = 2,4,6-trinitrobenzaldehyde test.

5-n-alkyl group	Glucose-6-phosphate dehydrogenase: Thunberg 10^{-3}M	Thunberg 10^{-4}M	Thunberg 10^{-5}M	Manometric	Glyoxalase	Xanthine oxidase	D-Amino-acid oxidase	Carbonic anhydrate	TNB
Methyl	1·0	—	—	—	—	—	—	—	23
Ethyl	1·0	—	—	—	7	17	11	18	45
Propyl	1·4	—	—	0	14	10	—	24	52
Butyl	>4	1·0	—	—	23	0	11	40	67
Pentyl	>4	1·1	—	0	54	13	46	49	64
Hexyl	>4	1·1	—	—	57	8	71	57	75
Heptyl	—	>4	1·0	14	73	12	100	63	72
Octyl	—	>4	1·0	23	78	42	100	73	83
Nonyl	—	>4	1·1	62	85	84	100	90	76
Decyl	—	>4	1·5	77	80	51	100	52	84
Dodecyl	—	>4	1·3	77	19	10	92	62	80
Tetradecyl	>4	1·1	1·0	0	0	12	96	36	67
Hexadecyl	>4	1·6	1·2	0	0	10	86	—	42
Octadecyl	>4	1·1	—	0	0	—	83	—	28
Myristate	>4	1·3	—	—	14	0	53	58	60

Confirmation by direct spectrophotometric measurement of the rate of NADPH formation was not possible due to strong absorption by the drugs at 340 nm, but since they roughly doubled the time needed for bleaching of brilliant cresyl blue (0·1 μmol) by NADPH (0·5 μmol) in the enzyme free system at 10^{-4}M, and preserved the colour for long periods at 10^{-3}M, it was inferred that the inhibition was an artefact due to formation of drug-dye complexes. In support of this conclusion, malic dehydrogenase was also apparently inhibited to the same degree by the decyl derivative in a Thunberg system. Solutions of excess drug with brilliant cresyl blue slowly turned purple and deposited a black amorphous powder during several days. The supernatant remained pale blue but this residual colour was attributed to impurities since the dye could readily be separated into at least six components on a cellulose column. Lauric, myristic and palmitic acids, and lauryl sulphate (10^{-3}M) were weak inhibitors of the system, though inactive by spectrophotometric assay.

Colour change with or without precipitation, or failure to function as H-acceptor in association with the salicylates, or both, were noted for seven other commercial dyes of the dibenz-1,4-oxazine, -thiazine and diazine type but not with celestine blue B, pyronin G or 2,6-dichlorophenolindophenol. Salicylate (10^{-3}M) did not interfere with the assay of rat liver succinic dehydrogenase using the diazine, phenazine methosulphate, as H-acceptor. The inhibition of xanthine oxidase by the alkylsalicylates was not reversed by added flavin adenine dinucleotide.

Peak inhibitory activity was found at the decyl derivative when glucose-6-phosphate dehydrogenase was assayed manometrically using ferricyanide as H-acceptor (Table 2).

It was concluded that there was a weak inhibition of glucose-6-phosphate dehydrogenase by 5-n-alkylsalicylates but this was increased in the anaerobic system by the formation of drug-dye complexes. Potent anti-inflammatory agents also inhibited glucose-6-phosphate dehydrogenase at 10^{-3}M in the Thunberg assay (Table 3).

Table 3. *Inhibition of enyzmes by anti-inflammatory drugs.* TNB = 2,4,6-trinitrobenzaldehyde test. Drugs are at 10^{-3}M unless indicated otherwise. Values for the Thunberg method are ratios of test to control times for dye bleaching. (1 = inactive). Values elsewhere are % inhibition.

Drug	Glucose-6-phosphate dehydrogenase: Thunberg 10^{-3}M	Glucose-6-phosphate dehydrogenase: Thunberg 10^{-4}M	Glucose-6-phosphate dehydrogenase: Manometric	Glyoxalase	Xanthine oxidase	Carbonic anhydrase	D-Amino-acid oxidase	TNB
Indomethacin	>4	1·8	12	65	30	15	48	56
Flufenamic acid	>4	2·2	27	65	75	0	60	61
Phenylbutazone	1·8	1·2	61	18	7	15	76	37
Ibufenac	1·4	—	0	0	0	0	0	27
Salicylate	0[3]	—	0	0[1]	0[2]	10	18	18
Glycyrrhetinic acid	1·7	—	0	43	0	18	21	10
Phenazone	1·4	—	0	19	0	0	10	7

[1] 0 at 10^{-2}M. [2] 37 at 10^{-2}M. [3] with preincubation 30 min/37°, 2·0.

Inhibition of glyoxalase by anti-inflammatory agents. In a different approach to anti-inflammatory testing, Whitehouse (1967) used methylglyoxal as a reagent. Table 3 shows that potent anti-inflammatory agents inhibited the conversion of methylglyoxal to lactate by rabbit liver glyoxalase. Milk xanthine oxidase, rat erythrocyte carbonic anhydrase and pig kidney D-amino-acid oxidase were also inhibited. By contrast with specific histidine decarboxylase, the pyridoxal dependent glutamic decarboxylase of guinea-pig brain was not inhibited up to 10^{-3}M concentration.

In contrast to xanthine oxidase and carbonic anhydrase, glyoxalase was completely inhibited by TNB at 10^{-3}M (80% at 10^{-4}M, 0 at 10^{-5}M). Bovine albumen tended to reverse the inhibition of glyoxalase by indomethacin but further investigation of this finding was not informative since the protein inhibited the enzyme at high concentrations.

The anti-glyoxalase test, like the TNB test, was found to give too many false positives when seeking anti-inflammatory activity in new compounds. The test and the TNB test were not complementary in the manner sought for. There was indeed a striking parallel between inhibitory potency in four *in vitro* tests and the chain lengths of 5-n-alkylsalicylates (Table 2). It could be reasonably concluded that the salicylates, acidic anti-inflammatory agents, and TNB can occlude from other reagents, the basic side chains of lysyl and arginyl residues in proteins, whether these be albumen or enzymes. This could occur in a non-specific manner and so include those special instances where a particular arginine or lysine residue is essential to biological activity. All the enzymes examined are concerned with transformation of acids. By inference, arginine or lysine residues, or both, may be involved in anti-inflammatory activity or inflammation. It is notable, however, that bradykinin (2 μg), a α,ω-*bis*-guanidine gave no trace of colour with TNB. It is interesting that while association with blood

proteins is usually considered to be detrimental to drug activity, the TNB test, a measure of drug-protein association, was proposed as a measure of pharmacological activity. This factor is a possible reason why the 5-n-alkylsalicylates had no anti-inflammatory activity. They are known to form stronger associations with albumen than does salicylate (Davison & Smith, 1961) and this was confirmed in preliminary experiments with the 5-n-decyl derivative.

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