Inhibition of Carbonic Anhydrase by Sulphonamides

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Mann & Keilin (1940) discovered that sulphanilamide and certain related substances are specific inhibitors of carbonic anhydrase. In subsequent work on this enzyme (reviewed by Davenport, 1946) these inhibitors have proved useful tools in the study of the physiological action of carbonic anhydrase. According to Mann & Keilin the NH_2 group of sulphanilamide is not concerned with the inhibitory action, for benzenesulphonamide and N^4 -acetyl-sulphanilamide are also powerful inhibitors. In contrast the SO_2NH_2 group is concerned, for no inhibitions were found with sulphanilamide derivatives of the type $R\cdot\text{SO}_2\text{NH}\cdot R'$ to which most of the sulphonamide drugs belong.

Relatively little is known on the quantitative comparison of the inhibitory powers of different sulphonamides. Davenport (1945) has shown that in dilute solutions of carbonic anhydrase the expression

$$\frac{\text{uninhibited fraction of carbonic anhydrase}}{\text{inhibited fraction of carbonic anhydrase}} \times \text{concentration of inhibitor}$$

is constant, which is to be expected if the inhibition is due to a simple reversible reaction between carbonic anhydrase and the inhibitor. Davenport determined the value of this expression (K , representing the inhibitor concentration when the inhibition is 50%) for sulphanilamide and for thiophene-2-sulphonamide in dilute enzyme solutions, by measuring the inhibition at different concentrations of the inhibitor. For sulphanilamide, K was $1.0 \times 10^{-6} \text{ M}$ at 0° and $1.8 \times 10^{-6} \text{ M}$ at 10° ; for thiophene-2-sulphonamide it was $1.6 \times 10^{-7} \text{ M}$ at 0° and about the same at 10° .

This paper is an account of a survey of the inhibitory power of twenty-five sulphonamides tested in connexion with the experiments on carbonic anhydrase described by Krebs & Roughton (1948). The concentrations causing an inhibition of 50% in various conditions were ascertained.

EXPERIMENTAL

The inhibition of the reaction between bicarbonate and phosphate buffer was measured with the technique described by Krebs & Roughton (1948). The carbonic anhydrase preparation was a chloroform-ethanol extract of ox blood containing 9.2 mg. dry matter/ml. For the test

it was diluted 80 or 160 times and 0.1 ml. of the diluted solution, containing 0.012 or 0.006 mg. dry matter, was added to the main compartment. The inhibitor, in a volume of 0.1 ml., was added to the main compartment. Preliminary experiments established the order of magnitude required to produce a 50% inhibition and several measurements were then made at the critical inhibitor concentration. Davenport's formula was used in calculating the concentration causing an inhibition of 50%. As pointed out by Roughton & Booth (1946) and Krebs & Roughton (1948) results often show considerable variations when the concentrations of carbonic anhydrase are very low, as was the case in the present experiments. All data given are based on at least three independent measurements but no great accuracy can be claimed. In general, the data obtained at 0° are more reliable than those obtained at 15° . The error at 0° is estimated at $\pm 30\%$.

RESULTS

Data obtained with the foregoing procedure are given in Table 1. At 15° the inhibiting power was, in all cases, lower than at 0° , the differences between the values at the two temperatures varying from substance to substance. The data for sulphanilamide agree well with Davenport's (1945) figures, whilst in the case of thiophene-2-sulphonamide the present figures are higher than those recorded by Davenport. The most powerful inhibitors were prontosil red and prontosil soluble which caused 50% inhibition at a molar concentration of about 10^{-8} . At 0° *p*-toluenesulphonamide, acetylsulphanilamide, *p*-sulphonamidobenzoic acid, naphthalene-1-sulphonamide, naphthalene-2-sulphonamide and 2-chloropyridine-5-sulphonamide caused a 50% inhibition at molar concentrations between 1×10^{-7} and 2×10^{-7} ; they are thus more powerful inhibitors than thiophene-2-sulphonamide. The comparison of several *p*-substituted benzenesulphonamides shows that in this series the inhibiting power decreased in the following order:

p-Toluenesulphonamide was more active as an inhibitor than *o*-toluenesulphonamide; N^4 -acetylsulphanilamide was more active than sulphanilamide, and benzenesulphonamide much more active

Table 1. *Inhibition of carbonic anhydrase by sulphonamides*

Substance	Formula	Concentration causing 50% inhibition (10^{-7} M)	
		At 0°	At 15°
A. <i>p</i> -Substituted benzenesulphonamides:			
Benzenesulphonamide	. SO_2NH_2	4.6	—
<i>p</i> -Toluenesulphonamide	CH_3 · . SO_2NH_2	1.0	2.4
<i>p</i> -Aminobenzenesulphonamide (sulphanilamide)	NH_2 · . SO_2NH_2	9.0	19
<i>p</i> -Acetamidobenzenesulphonamide (<i>N</i> ⁴ -acetylsulphanilamide)	$\text{CH}_3\cdot\text{CO}\cdot\text{NH}$ · . SO_2NH_2	1.2	2.0
<i>p</i> -Aminomethylbenzenesulphonamide (marfanil)	$\text{NH}_2\cdot\text{CH}_2$ · . SO_2NH_2	25	—
<i>p</i> -Sulphonamidobenzamidine	$\text{NH}_2(\text{NH})\text{C}$ · . SO_2NH_2	41	—
<i>p</i> -Sulphonamidobenzamidoxime	$\text{NH}_2(\text{NOH})\text{C}$ · . SO_2NH_2	11	—
<i>p</i> -Sulphonamidobenzoic acid	COOH · . SO_2NH_2	2.0	2.7
B. Other aromatic sulphonamides:			
<i>o</i> -Toluenesulphonamide	. CH_3 . SO_2NH_2	6.3	7.7
<i>o</i> -Sulphonamidobenzoic acid	. COOH . SO_2NH_2	5.3	15
Toluene- <i>o</i> -sulphonamide	. $\text{CH}_2\cdot\text{SO}_2\text{NH}_2$	150	—
Naphthalene-1-sulphonamide	. SO_2NH_2	1.6	2.0
Naphthalene-2-sulphonamide	. SO_2NH_2	1.0	2.7
Thiophene-2-sulphonamide	. SO_2NH_2	3.0	3.8
2-Chloropyridine-5-sulphonamide	. SO_2NH_2 Cl. N	1.2	—
4-Sulphonamido-2':4'-diaminoazobenzene (prontosil red)	—	0.06	—
4'-Sulphonamidophenyl-2-azo-7-acetamido- 1-hydroxynaphthalene-3:6-disulphonic acid (Na salt) (prontosil soluble)	—	0.10	—
C. <i>N</i> ¹ -Substituted sulphonamides:			
Sulphamezathine	—	2,200	4,200
Sulphapyridine	—	510	1,400
Sulphathiazole	—	6,600	7,700
Sulphaguanidine	—	300	650
Sulphacetamide	—	6,500	19,000
Sulphanilyl sulphanilate	—	12,000	21,000
<i>p</i> -Toluenesulphonylglycine	CH_3 · . $\text{SO}_2\text{NH}\cdot\text{CH}_2\cdot\text{COOH}$	4,400	7,600
D. Aliphatic sulphonamide:			
β -Cyanoethanesulphonamide	$\text{CN}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{SO}_2\text{NH}_2$	380	—

than toluene-*o*-sulphonamide. The one aliphatic sulphonamide which was tested was a relatively weak inhibitor.

Sulphonamide drugs of the type $R.SO_2NH.R'$ (sulphapyridine, sulphathiazole, sulphamezathine and sulphaguanidine) also inhibited carbonic anhydrase when high concentrations were tested. The concentrations required to produce a 50% inhibition were between 100 and 10,000 times higher than in the case of analogous compounds without a substituent attached to the N^1 atom. But even though the inhibitory capacity of these substances is smaller than that of other sulphonamides they have marked effects in molar concentrations of 10^{-3} or 10^{-4} . For example, sulphapyridine causes 73% inhibition (at 15°) when the molar concentration is 2×10^{-4} (or 5.2 mg./100 ml.). These are concentrations which are reached in the blood plasma in the standard procedures of treatment. Much higher concentrations may occur in the urine and, if the drug is given orally, in the stomach and in the intestinal tract.

Inhibition of carbonic anhydrase in the presence of tissue constituents

The inhibition of carbonic anhydrase by a given concentration of inhibitor depends on the affinity of the inhibitor for the enzyme and for other substances in the solution (see Davenport, 1941; van Goor, 1946). In the very dilute solutions of carbonic anhydrase to which the measurements recorded in Table 1 refer the second factor was of minor importance. Animal tissues and body fluids may be expected to contain substances capable of combining with sulphonamides and the inhibition of carbonic anhydrase in its natural medium may therefore be smaller than the values given in Table 1 suggest. The following experiments were undertaken to assess the order of magnitude of the effect of tissue constituents on the inhibition of carbonic anhydrase.

Sheep kidney cortex was homogenized in a Waring Blender with 1 vol. of 0.1M-phosphate buffer of pH 7.0. The homogenate was heated for 1 hr. in boiling water to inactivate carbonic anhydrase (heating to 70° proved insufficient). The heated material was mixed with an equal volume of 0.02M-phosphate buffer of pH 7.0 and again homogenized. The resulting liquid contained 5.2% dry matter and had a pH of 7.0. For carbonic anhydrase experiments 2 ml. of the homogenate were placed in the main compartment of a Warburg cup, together with 0.2 ml. additional solutions (carbonic anhydrase, inhibitor solution, or water). The side-arm contained 0.2 ml. 0.25M- $NaHCO_3$. After mixing, a total pressure of about 200 mm. was observed and experiments with inhibitors indicated that the heated homogenate still contained a measurable amount of carbonic anhydrase. To test the effect of inhibitors 0.1 ml. of a diluted (1:160) chloroform-ethanol extract of ox blood was added to the main compartment, with and without the inhibitor to be examined.

The data obtained are given in Table 2. It will be seen that the concentration required to produce a 50% inhibition is in all cases increased by the presence of tissue homogenate. The extreme cases were those of prontosil red, where the concentration was 83 times, and *p*-sulphonamidobenzoic acid where it was only 1.2 times as high as that required in the absence of tissue homogenate (at 0°).

DISCUSSION

Inhibitory effect in warm-blooded animals under physiological conditions. Whilst the inhibitory action of the sulphonamides decreased in all cases with rising temperature, the temperature coefficient varied considerably from substance to substance. The manometric technique is not suitable for the exact measurement of the inhibition at body temperature owing to the relatively high rate of the uncatalyzed reactions of carbonic acid at that temperature, but an approximate estimate of the inhibition can be obtained by extrapolation from the

Table 2. *Effect of tissue homogenates on the inhibition of carbonic anhydrase by sulphonamides*

For experimental conditions, see text. The effect of tissue constituents is indicated by the ratio

$$\frac{C_H}{C_0} = \frac{(\text{concentration of sulphonamide causing 50\% inhibition with homogenate})}{(\text{concentration of sulphonamide causing 50\% inhibition without homogenate})}$$

Substance	Experiments at 0°		Experiments at 15°	
	C_H (10^{-7} M)	Ratio C_H/C_0	C_H (10^{-7} M)	Ratio C_H/C_0
Sulphanilamide	39	4.3	—	—
<i>N</i> ⁴ -Acetylsulphanilamide	5.4	4.5	11	5.5
<i>p</i> -Toluenesulphonamide	7.0	7.0	12.7	5.3
<i>p</i> -Sulphonamidobenzoic acid	2.3	1.2	3.7	1.4
Thiophene-2-sulphonamide	8.0	2.7	8.0	2.1
Naphthalene-1-sulphonamide	10	8.3	—	—
Prontosil red	5	83	18	—
Sulphaguanidine	2300	7.7	—	—

data given for 0 and 15°. The effect of tissue constituents on the inhibition under physiological conditions will likewise have to be assessed by extrapolation. The measurements listed in Table 2 were carried out in a medium containing about one quarter of the amount of tissue material present in the intact organ. Moreover, the protein was denatured by heat treatment.

Choice of inhibitor for physiological experiments. Of the substances tested *p*-sulphonamidobenzoic acid may have advantages over other substances in inhibitor experiments. Its powerful action is not much affected by temperature and by the presence of tissue constituents. The neutral sodium salt is very soluble in water (about 20 % w/v) and can therefore be added in concentrated solution. It is worthy of note that *p*-sulphonamidobenzoic acid has been found in urine after administration of marfanil (Hartles & Williams, 1946) and of *p*-toluenesulphonamide (Flaschen-träger, Bernhard, Löwenberg & Schläpfer, 1934). In some experiments it may be disadvantageous that

the inhibiting principle is contained in an anion, as in this substance; in such cases benzene-, *p*-toluene-, naphthalene- or thiophene-2-sulphonamide may be preferable.

SUMMARY

1. The inhibitory action of twenty-five sulphonamides on carbonic anhydrase was examined under varying conditions. Data are given for the concentration causing an inhibition of 50 % at 0 and at 15°, in dilute enzyme solution, and also in the presence of tissue constituents.

2. Relations between chemical constitution and inhibitory power and the suitability of various substances for inhibitor experiments are discussed.

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Studies in the Biochemistry of Micro-organisms

79. FUSCIN, A METABOLIC PRODUCT OF *OIDIODENDRON FUSCUM* ROBAK PART 1. PREPARATION, PROPERTIES AND ANTIBACTERIAL ACTIVITY

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Smith (1946), in a note on the occurrence of species of the micro-fungus *Oidiodendron* Robak in Britain, states that 'the genus *Oidiodendron* was erected by Robak (1932) on three species which were all found growing on ground wood pulp in Norwegian mills. The original three species are *O. nigrum*, *O. fuscum* and *O. rhodogenum*. Later, Robak (cited by Melin & Nannfeldt, 1934, p. 440) described a fourth species, *O. griseum*, and a further addition to the genus was made by von Szilvinyi (1941) with his *O. flavum*.'

The purpose of the present communication is to describe the preparation, properties and antibacterial activity of a hitherto undescribed mould metabolic

product, isolated from two different strains of *O. fuscum*, for which the name *fuscine* is proposed. Both the strains were identified by Mr G. Smith and were isolated originally from British sources, one—strain Ag. 112—by the late J. H. V. Charles from gun cotton at Ardeer, Scotland, in 1926, the other—strain Camb. 111—by Dr Alan Burges in Cambridge, England, in 1945, from the surface humus in a pine plantation.

It has been found that, when either of these strains is grown on Czapek-Dox 5 % glucose solution, the yellow-orange culture filtrate after 28–30 days' incubation at 24° shows a marked antibacterial activity against *Staphylococcus aureus*. Aeration of