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Reactivity–Selectivity Correlations

I. The Reactivity of Alkyl Aryl Sulfates Towards Papain and Ficin[1]

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A series of alkyl aryl sulfates has been investigated as inhibitors of papain and ficin activity. The results show that the percentage of residual enzymatic activity depends on several factors, including the solvolytic reactivity of the alkylating agent. It is possible to correlate the reactivities of the sulfates with a selectivity parameter which is based on product ratio results. The resulting correlations indicate that the reactivity–selectivity principle is applicable to this system. It is shown, however, from the results of amino acid analyses that specific methylation of active-site cysteine or histidine residues is not effective with the enzymes in the native or denatured state. This phenomenon contrasts with reported work on methyl *p*-nitrobenzenesulfonate in which extensive methylation of papain in the denatured state was observed. The finding that the nonspecific methylation of the enzymes by the alkyl aryl sulfates leads to inhibition of enzymic activity is discussed in terms of conformational and other effects.

INTRODUCTION

Chemical modification of enzymes is a widely accepted procedure which can provide useful information about the structure and mode of action of enzymes (*1–4*). Of the various possible modification reactions, acylation and alkylation are among the most often employed and can be specially useful in affording covalent labels of the active sites. The underlying principle in such procedures is that, in general, functional groups participating in the active site of an enzyme have enhanced reactivities. However, simple alkylating agents such as methyl iodide show little, if any, selectivity in reaction with the various functional groups present in enzymes.

We observed in our first study with methyl *p*-nitrophenyl sulfate (*5*) that this compound reacted with methanol and methoxide ion exclusively by alkyl–oxygen scission. Subsequently we found that other nucleophiles, including those with primary amino and mercapto functionalities, likewise react specifically at the alkyl carbon

[1] Bond Scission in Sulfur Compounds, Part XII. For Part XI see E. Buncel, J. P. Millington, and J. F. Wiltshire, *Canad. J. Chem.*, **55**, 1401 (1977).

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centre of methyl *p*-nitrophenyl sulfate (*6–8*). We have drawn attention (*5*) to the reported use of the structurally related methyl *p*-nitrobenzenesulfonate for the chemical modification of enzymes (*9*, *10*) suggesting that alkyl aryl sulfates might also be effective for this purpose.

An interesting aspect of chemical modification studies is the relationship between reactivity and selectivity. This requires the availability of a suitable family of modifying reagents in which structural changes along the series can be correlated with reactivity parameters. The family of alkyl aryl sulfates, with various substituents in the phenyl moiety, appear to be highly suitable as a series of modifying agents, enabling one to evaluate reactivity–selectivity correlations. In the present paper we discuss our results obtained on investigating the reactivities of these sulfates towards the enzymes papain and ficin.

These enzymes belong to the cysteine proteinases and depend for their enzymic activity on the sulfhydryl function of a free cysteine residue. Other cysteine residues are also present as disulfide groupings. The structure of papain is now established and its mechanism of action has been elucidated in a number of studies (*11*, *12*). Though ficin has been less extensively investigated, it is believed to have a structure homologous to papain and to hydrolyse peptide bonds by a similar mechanism (*12*). The special properties of the active site in papain are based largely on interaction between the sulfhydryl group of cysteine-25 and the imidazole group of histidine-159. Several subsites are believed to be associated with the active site. Papain exhibits a broader specificity in comparison with serine proteinases, such as chymotrypsin, and this will be shown to be significant in the present work. In previous work on the alkylation of papain by methyl *p*-nitrobenzenesulfonate (*10*) it was found that the enzyme in the denatured state was completely deactivated by this reagent and that there was 75% methylation of one cysteine residue, presumed to be cysteine-25. Reaction of the alkylating agent with the enzyme in the native state resulted in no loss of activity, and there was no methylation of cysteine or histidine residues.

EXPERIMENTAL

Preparation of Aryl Methyl Sulfates

The sulfates used in the present work correspond to the structure $X–C_6H_4–OSO_2OCH_3$, where X is *p*-NO_2, *m*-NO_2, *p*-Br, H, and *p*-CH_3. All of these were obtained by reaction of dimethyl sulfate with the appropriately substituted benzenediazonium salts following the general procedure which is described below (see also Ref. (*13*)).

The diazonium tetrafluoroborate salts were prepared from the corresponding substituted anilines as described by Flood (*14*). The diazonium salts were allowed to react with dimethyl sulfate according to the method described previously (*13*). For the *p*-nitro-, *m*-nitro-, and *p*-bromo-substituted compounds, the reaction was run at 50°C for 10 days, while for the other two compounds the reactions were allowed to occur at room temperature for 2 days. The reaction mixture was then continuously extracted with petroleum ether (40–60°C) for 48 hr using a soxhlet apparatus. After evaporation of the petroleum ether the excess dimethyl sulfate was distilled under vacuum. Subsequent crystallization for the *p*-nitro- and *m*-nitro-substituted phenyl methyl

TABLE 1

Compound ($X-C_6H_4-OSO_2OCH_3$) (X)	Elemental analysis						Physical properties, BP or MP
	C(%)		H(%)		S(%)		
	Calcd	Found	Calcd	Found	Calcd	Found	
p-CH_3	47.55	47.48	4.95	5.02	15.85	15.82	bp 110–116°C/0.5 mm
H	44.63	44.89	4.25	4.04	17.10	17.17	bp 98–104°C/0.5 mm
p-Br	31.46	32.34	2.62	2.71	11.99	11.57	mp 3–4°C, bp 117–125°C/1 mm
m-NO_2	36.07	35.70	3.03	3.15	13.76	13.92	mp 43°C
p-NO_2	36.07	35.86	3.03	3.18	13.76	—	mp 45°C

sulfates, or distillation at low pressure for the other derivatives, yielded the pure compounds. The analytical data and physical properties are given in Table 1.

Stock solutions of the sulfates were prepared in anhydrous diethyl ether, in a concentration of 3.7×10^{-2} *M*. The sulfates are stable in ethereal solution for prolonged periods when kept at 0°C with the exclusion of moisture.

Determination of Solvolysis Rate Constants of the Aryl Methyl Sulfates

The solvolytic reactions were carried out by addition of 10–50 μl (3.7×10^{-2} *M*) of a stock solution of the substituted aryl methyl sulfate in ethyl ether to 3 ml of aqueous methanol (30% MeOH, v/v). The reactions were monitored spectrophotometrically, either by means of repeated scanning between 240 and 320 nm, or at a constant wavelength corresponding to the maximum absorption of the reaction product, $X-C_6H_4-OSO_3^-$. The first procedure provides additional information regarding isosbestic behavior, while the second procedure makes available more data for the graphic determination of rate constants. In all cases, a plot of log ($OD_\infty - OD_t$) vs time was linear for at least three half-lives.

Preparation of the Enzymes

Papain and ficin were obtained in the form of mercuric derivatives from crude preparations (Sigma Chemical Co.) using the method of Sluyterman and Wijdenes (*15*). They were stored as solutions in 10^{-2} *M* sodium acetate (pH 6.5) and before use further purified by passage through Sephadex-G25 (Pharmacia Ltd., 1.5 × 25-cm columns). Disc gel electrophoresis (*16*) showed that the papain so obtained was homogeneous, while the ficin consisted of several active components of essentially identical composition (*17*).

The molarity of the enzyme solutions was calculated from the observed optical density at 280 nm, assuming the same ε value (50,000 M^{-1} cm^{-1}) for both enzymes, and taking a molecular weight of 23,400 for papain and 25,000 for ficin. The enzyme solutions, containing sodium acetate (10^{-2} *M*), had the concentrations 1×10^{-5} *M* in the case of papain and 7×10^{-5} *M* for ficin.

Determination of Enzyme Activity

Enzymatic activities were assayed using *N*-carbobenzoxyglycine *p*-nitrophenyl ester (CGN, Sigma Chemical Co.), the esterolysis catalysed by the proteases yielding the *p*-

nitrophenolate ion, the appearance of which was monitored at 410 nm. As the enzymes were present initially in the form of inactive mercuric derivatives, the presence of a thiol (2-mercaptoethanol, Eastman) was necessary to liberate the active forms; EDTA was added also.

The following stock solutions were prepared: CGN (9.8 mg/10 ml of acetonitrile), EDTA (111 mg/10 ml of sodium acetate solution, 1×10^{-2} *M*), 2-mercaptoethanol (10 μl ml of sodium acetate solution, 1×10^{-2} *M*).

The assay was performed by adding to each cuvette of 10-mm pathlength 2.7 ml of acetate solution, 0.1 ml of EDTA solution, 0.1 ml of mercaptoethanol solution, and 0.1 ml of CGN solution. The reaction was started by adding 0.1 ml of the enzyme solution to the sample cell, and the increase in absorbance at 410 nm was followed. The resulting absorbance-time curve was linear over the first ~40 sec. The slope of the linear portion provides a measure of the enzyme activity (Δ absorbance per minute).

Inactivation of Enzymes by Action of Aryl Methyl Sulfates

The reaction mixture consisted of 1 ml of the enzyme solution (in acetate solution), 0.5 ml of methanol, and a variable volume (maximum 0.1 ml) of 2-mercaptoethanol solution, 7.6×10^{-2} *M*. (The presence of methanol in the reaction medium was necessary to solubilize the ethereal solution of the aryl methyl sulfate.) In order to inactivate the enzyme, an aliquot (maximum of 80 μl, 3.7×10^{-2} *M*) of the ethereal solution of the sulfate was added. The enzyme essay was performed on 0.1-ml aliquots of the reaction solution, at various time intervals, using the CGN method described above.

Each run on enzyme inactivation by the aryl methyl sulfate was calibrated against a blank experiment which was performed under identical conditions but with omission of the sulfate. The reaction mixture in these control experiments contained the enzyme, 2-mercaptoethanol, methanol, and ether, in the same quantities as given above. Aliquots (0.1 ml) of the solutions were taken at different times and the enzyme activity was determined by the CGN method. The control experiment defines 100% activity and also provides a test of the stability of the enzyme in the reaction medium.

Hence the percentage inactivation of enzyme by the sulfate is given by

$$\% \text{ inactivation} = \frac{\text{enzyme activity in test experiment}}{\text{enzyme activity in control experiment}} \times 100.$$

The concentration of inactivated papain in a given test experiment is thus given by

$$\text{concn. of inactivated papain} = \text{initial concn. of papain} \times \% \text{ inactivation}/100.$$

Products of methylation experiments

(1) *Reaction of methyl p-nitrophenyl sulfate with native papain.* Mercurial papain was concentrated with Ficoll (Pharmacia Ltd.) and then passed through a G25-Sephadex column after treatment with mercaptoethanol. The enzyme obtained, in 10^{-2} *M* NaOAc, was treated with a tenfold molar excess of the sulfate as described above. After 75 min the reaction mixture was freeze-dried. Acid hydrolysis in 6-*N* HCl at 110°C for 22 hr under vacuum was followed by amino acid analysis. Elution times for *S*-methyl cysteine and for 1- and 3-methyl histidines were measured with authentic samples.

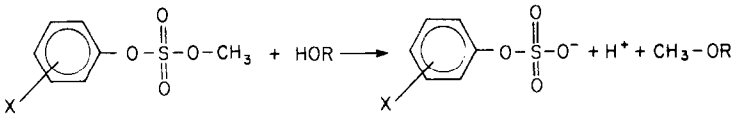
(2) *Reactions of reduced and denatured papain and ficin with methyl p- and m-nitro- and p-methylphenyl sulfates.* The enzymes were reduced with mercaptoethanol and

denatured in concentrated urea under a nitrogen atmosphere essentially by the method of Heinrikson (*10*). The sulfates were reacted in 100-fold excesses (mole per mole of enzyme) for 30 min, after which reaction mixtures were dialyzed against water, freeze-dried, and hydrolyzed as described above, and amino acid analyses were performed conventionally.

RESULTS

1. Solvolytic Rate Constants

The reaction corresponding to the solvolysis process is shown in the following equation, where ROH represents the solvent system used in the present work.



In Table 2 are listed the solvolytic rate constants (k_S) in 30% aqueous methanol at 25°C for the various aryl methyl sulfates used, and Fig. 1 shows the corresponding Hammett σ plot. The linearity of this plot indicates that the reactivity of these compounds is a function only of the substituent electronic effects as given by the σ values. The slope of the plot yields the Hammett ρ value, 0.67.

TABLE 2

SOLVOLYTIC RATE CONSTANTS (k_S) FOR THE ALKYL ARYL SULFATES IN 30% AQUEOUS METHANOL (v/v) AT 25°C

Sulfate	$10^4\, k_S$ (sec^{-1})
Methyl *p*-nitrophenyl sulfate	25.0
Methyl *m*-nitrophenyl sulfate	21.0
Methyl *p*-bromophenyl sulfate	10.0
Methyl phenyl sulfate	8.0
Methyl *p*-methylphenyl sulfate	5.5

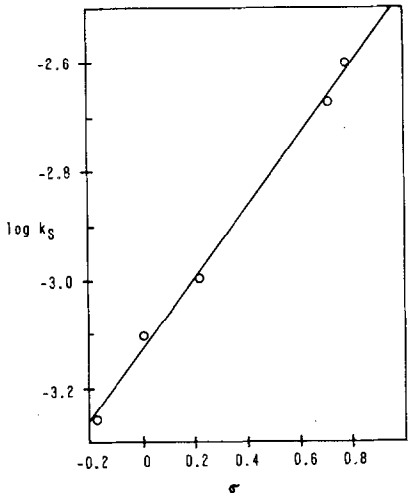


FIG. 1. Hammett σ-ρ plot for the solvolysis of methyl aryl sulfates in 30% aqueous methanol at 25°C.

2. Results for the Inactivation of Papain[2]

(*a*) *Effect of 2-mercaptoethanol concentration.* A dependence of the inactivation process on 2-mercaptoethanol concentration is expected, since papain is incubated as its mercury form and the thiol is needed to free the enzyme. After this process has occurred, the reaction between methyl *p*-nitrophenyl sulfate and the enzyme, leading to inactivation, can take place.

Table 3 shows the results of experiments in which papain was incubated with a constant concentration of methyl *p*-nitrophenyl sulfate while the concentration of 2-mercaptoethanol was varied. It is seen that the percentage inactivation increased with increasing concentration of 2-mercaptoethanol used. The concentration of 2-mercaptoethanol was not raised beyond the values given in the table so as to minimize the competitive reaction of this nucleophile with the aryl methyl sulfate. Quantitative aspects of the competitive processes are considered in the following section.

TABLE 3

EFFECT OF CHANGE OF CONCENTRATION OF 2-MERCAPTOETHANOL IN THE INACTIVATION OF PAPAIN (6×10^{-6} *M*) WITH METHYL *p*-METHYLPHENYL SULFATE (1.8×10^{-3} *M*) IN 30% AQUEOUS METHANOL SOLUTION CONTAINING SODIUM ACETATE (10^{-2}*M*) AT 25°C

[2-Mercaptoethanol] (*M*)	Residual activity (%)
0	90
4.75×10^{-5}	80
2.38×10^{-4}	49
4.75×10^{-4}	20

(*b*) *Effect of concentration of methyl-p-methylphenyl sulfate.* The results of experiments in which the sulfate concentration is varied while the concentrations of papain and of 2-mercaptoethanol are kept constant are shown in Table 4. It is seen that there is virtually no loss in activity when [sulfate] = 1.8×10^{-4} *M*, but the loss of activity is extensive when [sulfate] = 1.8×10^{-3} *M*. The latter condition was chosen for the subsequent work with the various sulfates (Tables 6–9).

The requirement for a relatively large sulfate concentration is explained by the nucleophilicity of the thiol functions in the system. The SH derives in part from papain (6×10^{-6} *M*) and in part from the mercaptoethanol (4.75×10^{-4} *M*), yielding [total SH] = 4.81×10^{-4} *M*. Hence we have, under the chosen operating conditions (Table 4, second entry), [sulfate]/[total SH] = 4 and [sulfate]/[enzyme] = 300. Under these conditions the reaction of enzyme with the sulfate can effectively compete with the other processes occurring in the system.

(*c*) *Stability of papain and ficin in 30% aqueous methanol.* The stability of papain and ficin was tested for each experiment by means of the control run described previously. The results, given in Table 5, show that there is no loss of enzyme activity

[2] All the experiments on inactivation of papain and ficin were performed using the mercuric derivatives of the enzymes.

TABLE 4

EFFECT OF CHANGE OF METHYL *p*-METHYLPHENYL SULFATE CONCENTRATION ON THE INACTIVATION OF PAPAIN (6×10^{-6} *M*) IN THE PRESENCE OF 2-MERCAPTOETHANOL (4.75×10^{-4} *M*) IN 30% AQUEOUS METHANOL–SODIUM ACETATE SOLUTION AT 25°C

[CH_3–*p*-C_6H_4–OSO_2OCH_3] (*M*)	Residual activity (%)
1.8×10^{-4}	98
1.8×10^{-3}	20

TABLE 5

STABILITY OF FICIN AND PAPAIN IN 30% METHANOL–ACETATE (10^{-2}*M*) MEDIUM AT 25°C

Enzyme	Time (min)	Activity (%)
Ficin	0	100
(5×10^{-5} *M*)	70	98
	110	99
Papain	0	100
(6×10^{-6} *M*)	100	99
	130	99

during the first ~100 min of incubation. These results rule out the possibility of enzyme inactivation by the solvent medium.

TABLE 6

RESULTS FOR THE INACTIVATION OF PAPAIN (6×10^{-6} *M*) BY THE VARIOUS SULFATES (1.8×10^{-3} *M*) IN THE PRESENCE OF 2-MERCAPTOETHANOL (4.75×10^{-3} *M*) IN 30% AQUEOUS METHANOL CONTAINING NaOAc (1×10^{-2} *M*) AT 25°C[a]

p-CH_3		H		*p*-Br		*m*-NO_2		*p*-NO_2	
Time (sec)	Activity (%)	Time (sec)	Activity (%)	Time (sec)	Activity (%)	Time (sec)	Activity (%)	Time (sec)	Activity (%)
70	79.2	60	46.7	60	60.0	60	70.0	70	79.0
300	70.8	260	35.0	220	60.0	300	66.5	300	77.0
530	58.3	450	33.3	650	60.0	600	67.0	600	77.0
750	50.0	1450	33.3	1200	53.0	1200	66.0	1200	76.0
960	42.5	∞	33.0	∞	53.0	∞	66.0	∞	76.0
1230	42.5								
2090	29.2								
2900	18.8								
∞	18.5								

[a] The data given in the table are for single experiments while the results shown in Fig. 2 represent the averaged values derived from two or more experiments.

(*d*) *Effect of changing substituents on the aryl methyl sulfates.* The series of substituted aryl methyl sulfates was tested toward papain under the conditions [sulfate]/[enzyme] = 300 and [sulfate]/[total SH] = 4. Representative results are given

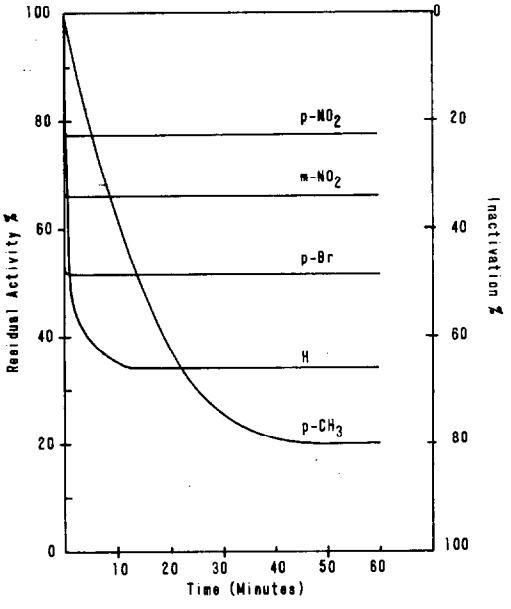


FIG. 2. Plots of residual activity vs time for the inactivation of papain by methyl aryl sulfates.

in Table 6, while in Fig. 2 is shown a plot of the percentage enzyme activity versus time for each substituted aryl methyl sulfate. It is seen that the percentage inactivation is dependent upon the reactivity of the aryl methyl sulfate.

TABLE 7

RESIDUAL ACTIVITY AT t_∞ FOR THE INACTIVATION OF PAPAIN (6×10^{-6} *M*) BY THE VARIOUS SULFATES (1.8×10^{-3} *M*) (AVERAGED VALUES AT t_∞) IN THE PRESENCE OF 2-MERCAPTOETHANOL (4.75×10^{-4} *M*) IN 30% AQUEOUS METHANOL-SODIUM ACETATE SOLUTION AT 25°C

Sulfate	Activity (%)
Methyl *p*-methylphenyl sulfate	20
Methyl phenyl sulfate	34
Methyl *p*-bromophenyl sulfate	52
Methyl *m*-nitrophenyl sulfate	66
Methyl *p*-nitrophenyl sulfate	77

In Table 7, the results of the individual experiments are presented showing the variation of percentage activity with time. Figure 2 was obtained from the averaged values of the duplicate experiments shown in Table 7.

3. Inactivation of Ficin with p-methyl- and m-Nitrophenyl Methyl Sulfate

While ficin was tested toward only these two substituted aryl methyl sulfates, the results (Table 8) clearly show that the percentage inactivation depends on the solvolytic reactivity of the aryl methyl sulfates. Thus methyl *p*-methylphenyl sulfate is more effective as an inactivator than methyl *m*-nitrophenylsulfate.

TABLE 8

INHIBITION OF FICIN (5×10^{-5} *M*) WITH METHYL *m*-NITROPHENYL SULFATE AND METHYL *p*-METHYLPHENYL SULFATE (1.8×10^{-3} *M*) IN THE PRESENCE OF 2-MERCAPTOETHANOL (4.75×10^{-4} *M*) IN 30% AQUEOUS METHANOL–SODIUM ACETATE SOLUTION AT 25°C

Sulfate	Residual activity at t_∞ (%)
Methyl *p*-methylphenyl sulfate	30
Methyl *m*-nitrophenyl sulfate	53

4. Methylation Results

(*a*) *Native papain.* No evidence of methylation on Cys or His residues was found.

(*b*) *Reduced and denatured papain and ficin.* The reaction of methyl *p*-nitrophenyl sulfate with papain yielded 20% of the theoretical *S*-methyl cysteine, while reactions of the other substituted sulfates produced less than 5% methylation of cysteine resides under the conditions discussed here.

The other product observed was 1-methyl histidine. From papain, yields of 40% of the two residues of histidine per molecule of enzyme were obtained with each sulfate employed. From ficin there was obtained a 30% yield of 1-methyl histidine using methyl *p*-methylphenyl sulfate and a 15% yield using methyl *m*-nitrophenyl sulfate.

DISCUSSION

The Nature of the Enzyme–Alkyl Aryl Sulfate Reactions

The results which we have obtained show that the enzymes papain and ficin are inactivated by the series of alkyl aryl sulfates studied. The efficacy of inactivation varies in a regular fashion along the series of sulfates employed. This inhibition of enzyme activity can be interpreted in terms of structure–reactivity correlations utilizing the reactivity–selectivity principle.

The methylation studies, performed with methyl *p*- and *m*-nitrophenyl sulfates and methyl *p*-methylphenyl sulfate, have shown that there is only slight *S*-methylation of the cysteine residues (maximum 20% for papain or ficin, see under Results for conditions). It is apparent that the alkyl aryl sulfates are not useful as specific alkylating agents for these enzymes. It is recalled that Heinrikson (*10*) obtained extensive methylation of a cysteine residue in papain in the reaction of the denatured enzyme with methyl *p*-nitrobenzenesulfonate, though no methylation was observed with the native enzyme. The contrasting behavior of the methyl aryl sulfates and of the methyl arenesulfonates is

noteworthy, and the underlying reasons provide further clues to the mode of enzyme action as well as reactivity–selectivity relationships.

Before discussing the significance of our results in terms of enzyme structure and reactivity, it is important to consider alternative explanations. One possibility would be that *S*-methyl cysteine, as formed by specific methylation of the Cys-25 residue, would be unstable under the acidic reaction conditions used in the amino acid analysis. However, the stability of *S*-methyl cysteine under the acidic conditions has been previously demonstrated (*10*), thus ruling out this possibility. In an alternative explanation of the loss of enzymatic activity, attack at the active thiol center would occur not at the carbon but at the sulfur atom of the aryl alkyl sulfates. Thus the cysteine residues would be specifically modified, but not methylated. However, no evidence for this reaction was found in the amino acid analyses. It is also noted that in our previous studies of the alkyl aryl sulfates with various nucleophilic species only alkyl–oxygen scission has been observed (*5–8*). The possibility of an indirectly induced inactivation process, dependent on the concentration of the aryl methyl sulfate rather than its structure, may also be considered. In that case, since the same concentration of aryl methyl sulfate was used in all the experiments, a constant percentage of inactivation would have been obtained throughout the series. However, the results clearly show a relationship between structure of the aryl methyl sulfate and the percentage inactivation, and this can only occur as a consequence of a direct interaction between the sulfates and the enzyme. These observations also eliminate the possibility that enzyme inactivation is caused by traces of some adventitiously present substances.

The nature of the inactivations effected by the sulfates examined here could have a number of possible factors as origin. A reasonable explanation is that methylation occurs at a variety of sites in the enzymes studied, for example, at amine centres on the outer surfaces of the proteins. Such substitution reactions, which need not be so extensive that they become apparent on conventional amino acid analysis of the enzymes, could seriously modify the tertiary structure of the proteins and so destroy their catalytic activity. Such destabilizing effects could be on the H-bonding or on the "hydrophobic" bonding interactions in the native enzymes. The decreased selectivity of the alkyl aryl sulfates versus the alkyl arenesulfonates toward cysteine residues in the enzyme can plausibly be explained on the basis of the higher reactivity of the former class of compounds.[3]

Related to the smaller degree of kinetic specificity noted above may be the somewhat broad substrate specificity which characterizes papain as compared to chymotrypsin, for example. The broad specificity of papain is indicated by the number and type of bonds which can be split by papain (e.g., in the A and B chains of insulin, the number of bonds split is 8 and 14, respectively) (*11*). This phenomenon may perhaps be connected with the nature of the active site in papain, namely, the presence of seven subsites, each of which can accommodate and bind an amino acid residue of the substrate (*12*). It is known, for example, that one of the subsites (S_2) specifically interacts with peptides containing a phenylalanyl residue, and the resulting products (ending with a free carboxyl group) act as powerful competitive inhibitors (*18*). It is possible, therefore, that

[3] Hydrolysis of methyl *p*-nitrobenzenesulfonate in 0.1 *M* sodium phosphate buffer at pH 7.94 and 25°C occurs with a rate constant $k_{obs} = 1.77 \times 10^{-4}$ sec^{-1} (*9*). We have found for the spontaneous hydrolysis of methyl *p*-nitrophenyl sulfate in 0.01 *M* NaOAc solution at 25°C, $k_{obs} = 26.3 \times 10^{-4}$ sec^{-1}. The rate ratio is therefore ~15, ignoring the difference in pH and salt concentrations.

in the present system the highly reactive sulfates may similarly modify one of the subsites, thereby leading to loss in catalytic activity.

It is to be expected that the various mechanisms considered above, and in particular the effect of random methylation on the H-bonding or hydrophobic bonding interactions, will interfere with the conformational requirements for catalytic activity and substrate specificity. The tertiary structure of papain entails two domains with a deep cleft between them, with the histidine-159 and cysteine-25 moieties on opposite side of the cleft, but in close proximity (*12*). This unique situation for the active site will be highly sensitive to changes in enzyme conformation discussed above.

Reactivity–Selectivity Relationships

Interpretation of the observed trends in the inhibition of enzyme activity, effected by the series of alkyl aryl sulfates, takes on added significance in the current situation of nonspecific alkylation. One wonders whether a nonspecific process can nevertheless lead to correlations explicable on the basis of electronic and other effects and whether selectivity relationships will be obeyed.

It follows from the data given under Results that the percentage inactivation at t_∞ is dependent upon the reactivity of a given aryl methyl sulfate. The percentage inactivation is related to the rate constant ratios of the competing reactions: a given aryl methyl sulfate is taking part in two competitive reactions, on the one hand, with the medium (solvolysis, k_S) and, on the other hand, with the added nucleophile (the enzyme, k_{Nu}). This situation is described by the equations [*1*] and [2], from which eq. [3] can be derived:

$$A + S \xrightarrow{k_S} A\text{-}S, \qquad [1]$$

$$A + Nu \xrightarrow{k_{Nu}} A\text{-}Nu, \qquad [2]$$

$$\frac{[A\text{-}S]}{[A\text{-}Nu]} = \frac{k_S[S]}{k_{Nu}[Nu]} . \qquad [3]$$

S represents the solvent system, A the aryl methyl sulfate, Nu the enzyme, A-S the product of the reaction with the solvent, and A-Nu the reacted inactivated enzyme. Equation [3] thus enables one to evaluate the rate constant ratio k_S/k_{Nu} from measurement of the product ratio [A-S]/[A-Nu], since the concentrations of the reagents is known.

The solvolytic reactivities of the aryl methyl sulfates (k_S) are given in Table 2. These rate constants give rise to a linear Hammett plot (Fig. 1), indicating that the reactivities of the aryl methyl sulfates are solely determined by electronic effects, as given by the σ substituent constants.

The percentage of inactivation of papain obtained with the various sulfates is shown in Fig. 2. It is seen that the percentage inactivation varies along the series of sulfates. This variation follows qualitatively the order of solvolytic reactivity of the corresponding aryl methyl sulfate: The more reactive the aryl methyl sulfate, the lower the percentage inactivation. It appears that the selectivity of the sulfates depends on their reactivity, as implied in the reactivity–selectivity principle (R.S.P.).[4]

[4] For extensive discussions of the reactivity–selectivity principle, including of systems which are apparent exceptions to this, see Refs. (*19*)–(*26*).

A quantitative approach to reactivity–selectivity relationship must include consideration of a reactivity parameter, a selectivity parameter, and the relationship between these (*19*). A suitable reactivity parameter is given by the solvolytic rate constants in the reaction series (k_S). The selectivity parameter should measure the ability of the reactant in question (that is the sulfate A) to discriminate between the different nucleophilic species (solvent S and enzyme Nu). This parameter is provided by the logarithm of the respective rate constant ratios, $\log k_S/k_{Nu}$. Hence, using Eq. [3], one obtains for the selectivity parameter in this system the relation in Eq. [4]. Now under the conditions of this study,

$$\text{Selectivity} = \log \frac{k_S}{k_{Nu}} = \log \frac{[Nu]\,[A\text{-}S]}{[S]\,[A\text{-}Nu]} \qquad [4]$$

with the solvent (S) in large excess and effectively constant in concentration, and with the sulfate in large excess over the enzyme (i.e., [A] $\gg$ [Nu]), the [A-S] term will also be effectively constant (see Table 9). Also, since the same value of the enzyme

TABLE 9

DERIVATION OF SELECTIVITY DATA AND COMPARISON WITH SOLVOLYTIC RATE CONSTANTS FOR INACTIVATION OF PAPAIN

Sulfate	[A-Nu][a] × 10^6 (M)	$\log \frac{1}{[A\text{-}Nu]}$ [b]	[A-S][c] × 10^3 (M)	k_S × 10^4 (sec^{-1})
Methyl *p*-nitrophenyl sulfate	1.38	5.86	1.799	25.0
Methyl *m*-nitrophenyl sulfate	2.04	5.69	1.798	21.0
Methyl *p*-bromophenyl sulfate	2.90	5.50	1.797	10.0
Methyl phenyl sulfate	3.96	5.40	1.796	8.0
Methyl *p*-methylphenyl sulfate	4.80	5.32	1.795	5.5

[a] The concentration of inactivated papain is calculated by multiplying the added concentration of papain (6×10^{-6} M) by the percentage inactivation and dividing by 100.

[b] Selectivity parameter as defined by Eq. [5].

[c] The concentration of product with the solvent system is calculated as the difference between the added concentration of sulfate (1.8×10^{-3} M) and the reaction product with papain [A-Nu].

concentration [Nu] is used throughout the series of experiments, the only variable term in Eq. [4] is [A-Nu], the concentration of the inactivated enzyme. Hence, [4] reduces to [5], where k is a proportionality constant:

$$\text{Selectivity} = \log \frac{k_S}{k_{Nu}} = k + \log \frac{1}{[A\text{-}Nu]}\,. \qquad [5]$$

A relationship between selectivity and reactivity might thus be expected in the form of a plot of log k_S (reactivity) versus log 1/[A-Nu] (selectivity). The results for [A-Nu] are included in Table 9, while Fig. 3 shows the plot of log 1/[A-Nu] vs log k_S. The plot is seen to have an approximately linear form, with a slope of 0.75. The linear relationship demonstrates that the R.S.P. is applicable to this system. We have studied previously the reactions of the same series of sulfates toward several nucleophiles in methanol, finding that the R.S.P. was likewise applicable (*8*).

Our finding of the validity of the R.S.P. in the inactivation of papain with the series of sulfates implies that an approach based on chemical reactivity alone can explain the observed data. This is in accord with our earlier conclusion that papain has no special affinity for the sulfates used. If the situation were otherwise, it would presumably be necessary to make use of more complex relationships (*27*) than the two-parameter equation employed. This situation is interesting, since even when the precise nature of the change undergone by the enzyme is not clear (e.g., a conformational change to a nonactive form), this change produces a loss of enzyme activity which follows a reactivity–selectivity pattern.

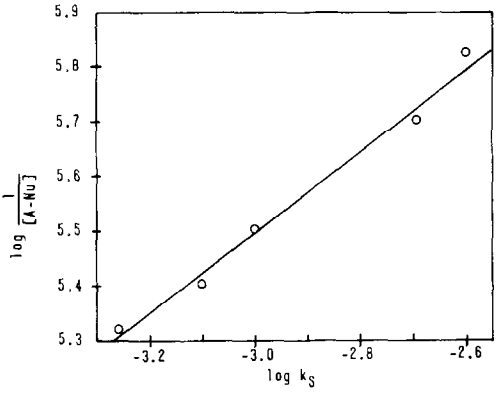


FIG. 3. Plot of log 1/[A-Nu] vs log k_S for the inhibition of papain by methyl aryl sulfates illustrating the reactivity–selectivity principle (Eq. [5]).

Our studies of the inactivation of ficin were more limited in extent, with only the *p*-methyl and *m*-nitrophenyl sulfates being tested with this enzyme. However, the results which we obtained (Table 8) point to conclusions similar to those in the case of papain.

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