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Studies on Chemical Carcinogens and Mutagens. XXXII.[1] Chemoselectivity of Alkylating Agents toward Pyridine in Aqueous Media—A Simple Procedure for Evaluation of Swain–Scott's Substrate Constants

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A simple method for the evaluation of the chemoselectivity of alkylating agents toward pyridine in aqueous media is proposed. The analytical method involves the evaluation of the relative rate of quaternization of pyridine with respect to the rate of hydrolysis in aqueous buffer (pH 6.0), *i.e.*, the evaluation of chemoselectivity toward pyridine in competitive nucleophilic attacks of a given alkylating agent on the nitrogen of pyridine *versus* the oxygen functions in phosphate buffer containing a large excess of pyridine. The term S_{py} is tentatively defined as $\log\{(N/(1-N))\times[H_2O]/[py]\}$, where N is the molar fraction consumed for pyridine quaternization after the complete consumption of the given alkylating agent, and $[H_2O]$ and $[py]$ are the concentrations of water and pyridine, respectively. S_{py} correlated linearly with the substrate constant (s) defined by Swain and Scott; $s=0.163S_{py}+0.209$ ($r=0.995$, $n=8$) at 37 °C in 4% acetone-containing 1/15 M phosphate buffer (pH 6.0). The dependences of the chemoselectivity on the reaction temperature and the acetone content of the medium are briefly discussed.

Keywords——alkylating agent; substrate constant; chemoselectivity; carcinogenicity; mutagenicity; genotoxicity; nitrosourea; methanesulfonate

There has been continuing discussion of the chemical characteristics of synthetic and environmental alkylating agents, since they are presumably important in the initiation step of induction of genotoxic end-points such as mutagenicity and carcinogenicity.[2-6] A representative expression of the chemoselectivity of alkylating agents is the substrate constant defined by Swain and Scott.[7]

$$\log(k_Y/k_w)=sn$$

where k_Y and k_w are the second-order rate constants for the reactions with nucleophile Y and with water, respectively, and n is the nucleophilic constant[7-9] which expresses the relative nucleophilic strength of a given nucleophile (Y) with respect to that of H_2O, and s is the substrate constant which expresses the relative sensitivity of a given alkylating agent, relative to CH_3Br, to changes in the nuclophilicity of the nucleophile. As we recently reported,[5,6,10] the chemoselectivity (S_{NBP}) toward 4-(p-nitrobenzyl)pyridine (NBP) in 60% acetone in 1/15 M phosphate buffer (pH 6.0) may be an alternative parameter; it was shown to correlate linearly with the Swain–Scott's substrate constant; $s=0.123S_{NBP}+0.318$ ($r=0.997$; $n=10$).

This paper describes another alternative expression of the chemoselectivity which is interconvertible to both the substrate constant defined by Swain and Scott[7] and the chemoselectivity toward NBP defined by us.[5] The present method involves the evaluation of the product ratio of the alkylated pyridine to the hydrolyzed alkylating agent in aqueous buffers containing a large excess of pyridine, *i.e.*, the evaluation of the ratio of the quaternization rate of pyridine (k_{py}) to the rate of hydrolysis. It turns out that this ratio is a measure of chemoselectivity in the competitive nucleophilic attacks of an alkylating agent on the nitrogen of pyridine *versus* the oxygen functions present in the aqueous reaction medium used in the present study. This method is different from the former two methods[5,7] in the following respects. (1) The experimental procedure is much simpler than that for Swain–Scott's substrate constant, which requires a series of kinetic measurements. (2) This method is preferable to the NBP method[5] in that analyses are possible in a wider range of reaction media, although the NBP method is superior in analyses with a smaller amount of the test sample.

Pyridine is readily soluble in water and most organic solvents, whereas NBP is soluble only in rather lipophilic media, so that the NBP method[5] was carried out only in 60% acetone-containing aqueous medium. (3) The NBP method is applicable only to agents which are extractable with organic solvents from the strongly alkaline aqueous medium but this method is effectively applicable to compounds containing acidic and/or hydrophilic functional groups.

In this paper, the chemoselectivities toward pyridine in aqueous media, denoted as S_{py}, are evaluated with several representative alkylating agents. It is shown that S_{py} is linearly correlated with Swain–Scott's substrate constant. The correlation equations were obtained at 37 °C in 4%, 20%, 40%, and 60% acetone-containing 1/15 M phosphate buffer (pH 6.0), and the temperature and solvent dependences of S_{py} are briefly described.

Materials and Methods

Materials——The alkylating agents examined in the present study were methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), isopropyl methanesulfonate (iPMS), ethyl tosylate (EtOTs), dimethyl sulfate (DMS), diethyl sulfate (DES), *N*-methyl-*N*-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea (ENU), and *N*-isopropyl-*N*-nitrosourea (iPNU), which were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) or synthesized by established methods.[5,6]

Measurements of Chemoselectivity Constants, S_{py}——A precisely weighed alkylating agent (usually 1—2×10^{-5} mol and in some cases *ca.* 10^{-4} mol) dissolved in an appropriate volume of acetone was combined with an appropriate volume of 1/15 M phosphate buffer (pH 6.0) containing 2.5×10^{-3} mol of pyridine at 37 °C in a glass tube. The ratios of acetone to the buffer in the test solutions were 1 ml/24 ml, 5 ml/20 ml, 10 ml/15 ml, and 15 ml/10 ml, respectively, *i.e.*, 4%, 20%, 40%, and 60% (v/v), respectively. The volumes of the reaction mixtures were 25.0, 24.7, 24.5, and 24.3 ml, respectively. The test tube was sealed and kept at 37 °C for a period long enough for the complete consumption of the alkylating agent tested. The end-point of the reaction was sometimes checked by means of a spot-test using NBP on a filter paper. Then, the reaction mixture was concentrated under reduced pressure and lyophilized. The residue was dissolved in a definite volume of water (usually 5—50 ml depending on how much alkylated pyridine was produced) and its ultraviolet (UV) absorption was measured at the absorption maximum of around 258 nm. The molar amount of the alkylated pyridine was calculated by referring to the molar extinction coefficient (ε) of the product previously determined;[11] 4370 at 259 nm for *N*-methyl-, 4710 at 259 nm for *N*-ethyl-, and 4370 at 258 nm for *N*-isopropyl-pyridine.

The chemoselectivity, denoted as S_{py}, is tentatively written as follows.

$$S_{py}=\log\left\{\frac{N}{(1-N)}\times\frac{[H_2O]}{[py]}\right\}$$

where n is the molar fraction consumed for pyridine quaternization after the complete consumption of the given alkylating agent, and $[H_2O]$ and [py] are the initial concentrations of water and pyridine, respectively.

The notations S_{py} (4), S_{py} (20), S_{py} (40), and S_{py} (60) are the S_{py} values determined in phosphate buffers containing 4%, 20%, 40%, and 60% acetone, respectively; [py] was 0.1 M in all the media examined and $[H_2O]$ was 53.3, 45.0, 34.0, or 22.9 M, respectively. The water concentrations were corrected by taking account of volume changes due to the mixing of the buffer and acetone.

$$S_{py}(4)=2.73+\log N/(1-N)$$

$S_{py}(20)=2.65+\log N/(1-N)$

$S_{py}(40)=2.53+\log N/(1-N)$

$S_{py}(60)=2.36+\log N/(1-N)$

Results

Chemoselectivity Constants, S_{py}, in Acetone-Containing 1/15 M Phosphate Buffer (pH 6.0) at 37 °C and the Correlation with Swain–Scott's Substrate Constant

The chemoselectivity constants of four alkyl sulfonates, two dialkyl sulfates, and three *N*-alkyl-*N*-nitrosoureas were measured in 4%, 20%, 40%, and 60% acetone-containing 1/15 M phosphate buffer (pH 6.0) at 37 °C. The results are shown in Table I together with Swain–Scott's substrate constants, *s*, and the values of chemoselectivity toward NBP, S_{NBP}. There is a linear correlation between S_{py} and *s* values at each acetone content. The correlation equations are shown below (*r*, correlation coefficient; *n*, number of the samples).

$s=0.163\ S_{py}(4)+0.209 \quad (r=0.995;\ n=8)$

$s=0.164\ S_{py}(20)+0.202 \quad (r=0.994;\ n=8)$

$s=0.168\ S_{py}(40)+0.196 \quad (r=0.991;\ n=8)$

$s=0.166\ S_{py}(60)+0.205 \quad (r=0.966;\ n=8)$

$S_{NBP}=1.198\ S_{py}(60)-0.368 \quad (r=0.994;\ n=8)$

Plots of the *s* value *versus* the S_{py} are shown in Fig. 1. The correlation becomes slightly poorer with increase in the acetone content of the reaction medium. This seems reasonable since the *s* values are averages obtained from reactions in aqueous media.

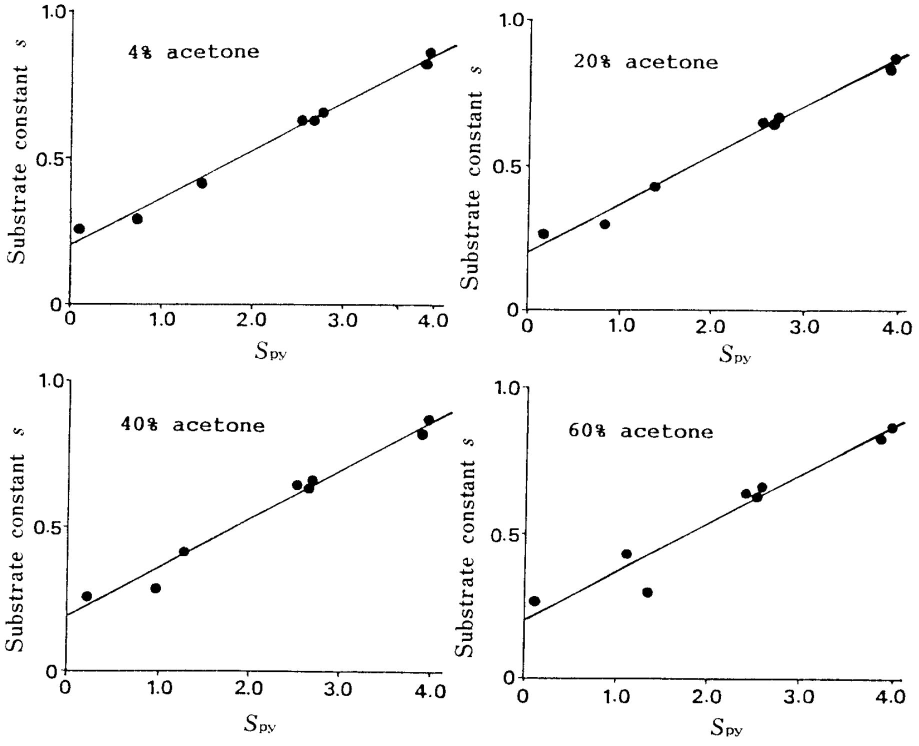

Fig. 1. Plots of Swain–Scott's Substrate Constants, *s*, *versus* S_{py} at 37 °C in 1/15 M Phosphate Buffers (pH 6.0) Containing 4%, 20%, 40%, and 60% Acetone

TABLE I. Chemoselectivity of Alkylating Agents toward Pyridine in Acetone–Phosphate Buffer (pH 6.0) at 37 °C

Compound	Acetone content (%)	$N \times 100$ (%)[a]	Chemoselectivity	
			S_{py}[b]	s[c]
Me-OSO_2CH_3 (MMS)	4	93.37 (±1.17)	3.86 (±0.06)	0.83 (0.84)
	20	95.04 (±1.24)	3.93 (±0.10)	(0.85)
	40	95.69 (±1.09)	3.87 (±0.10)	(0.85)
	60	97.04 (±0.26)	3.86 (±0.04)	(0.85)
Et-OSO_2CH_3 (EMS)	4	46.01 (±0.95)	2.66 (±0.01)	0.64 (0.64)
	20	51.48 (±0.78)	2.67 (±0.02)	(0.64)
	40	56.92 (±1.06)	2.64 (±0.02)	(0.64)
	60	59.46 (±0.32)	2.51 (±0.01)	(0.62)
iso-Pro-OSO_2CH_3 (iPNU)	4	0.97 (±0.14)	0.72 (±0.07)	0.29 (0.33)
	20	1.49 (±0.29)	0.83 (±0.10)	(0.34)
	40	2.60 (±0.48)	0.95 (±0.09)	(0.36)
	60	9.15 (±0.35)	1.35 (±0.02)	(0.43)
diMe-sulfate (DMS)	4	93.89 (±0.35)	3.91 (±0.02)	0.86 (0.85)
	20	95.27 (±0.35)	3.95 (±0.03)	(0.85)
	40	96.43 (±0.57)	3.95 (±0.08)	(0.86)
	60	97.62 (±0.07)	3.96 (±0.00)	(0.86)
diEt-sulfate (DES)	4	38.94 (±0.99)	2.53 (±0.02)	0.64 (0.62)
	20	44.05 (±0.12)	2.54 (±0.00)	(0.62)
	40	49.48 (±0.22)	2.51 (±0.00)	(0.62)
	60	53.63 (±0.02)	2.41 (±0.00)	(0.61)
Et-*O*-tosylate (EtOTs)	4	51.74 (±2.45)	2.76 (±0.05)	0.66 (0.66)
	20	54.05 (±1.59)	2.72 (±0.03)	(0.65)
	40	57.74 (±2.41)	2.66 (±0.04)	(0.64)
	60	61.90 (±0.11)	2.56 (±0.00)	(0.63)
Me-nitrosourea (MNU)	4	4.81 (±0.17)	1.43 (±0.02)	0.42 (0.44)
	20	5.04 (±0.18)	1.37 (±0.01)	(0.43)
	40	5.16 (±0.18)	1.26 (±0.02)	(0.41)
	60	5.48 (±0.19)	1.11 (±0.02)	(0.39)
Et-nitrosourea (ENU)	4	0.22 (±0.02)	0.07 (±0.04)	0.26 (0.22)
	20	0.32 (±0.04)	0.15 (±0.05)	(0.23)
	40	0.49 (±0.09)	0.21 (±0.08)	(0.23)
	60	0.56 (±0.03)	0.10 (±0.03)	(0.22)
iso-Pr-nitrosourea (iPNU)	4	0.19 (±0.03)	0.0063 (±0.00)	[d] (0.21)
	20	0.31 (±0.02)	0.14 (±0.03)	(0.22)
	40	0.50 (±0.03)	0.22 (±0.03)	(0.23)
	60	0.60 (±0.02)	0.13 (±0.02)	(0.23)

a) The molar fraction (%) of an alkylating agent consumed for quaternization of pyridine after the agent was completely consumed under the reaction conditions described in Experimental. *b*) Chemoselectivity of an alkylating agent toward pyridine at 37 °C in 1/15 M phosphate buffer (pH 6.0) containing 0.1 M pyridine and 4%, 20%, 40%, or 60% acetone. S_{py} is defined as $\log\{(N/(1-N)) \times [H_2O]/[py]\}$, where N is the molar fraction of the alkylating agent consumed for pyridine-quaternization, $(1-N)$ is the molar fraction consumed for hydrolysis, and $[H_2O]$ and [py] are the concentrations of water and pyridine, respectively, in the reaction medium. *c*) Swain–Scott's substrate constants reported in refs. 3, 4, and 7. The value in parentheses is that estimated from S_{py} by using the correlation equation in each reaction medium. *d*) Not reported.

Figure 2 shows the dependence of S_{py} of each agent on the Y-value, defined by Grunwald and Winstein,[12,13] which is a measure of the ionizing power of the reaction medium. Y values[12]: 3.417 for 4% acetone; 2.913 for 20% acetone; 1.981 for 40% acetone; 0.796 for 60% acetone. No appreciable dependence was found with most of the agents examined, except for MNU and iPMS; MNU shows a slight increase and iPMS shows a slight decrease with increase in the Y-value. The decrease indicates that the hydrolytic rate (k_{aq}) is more affected

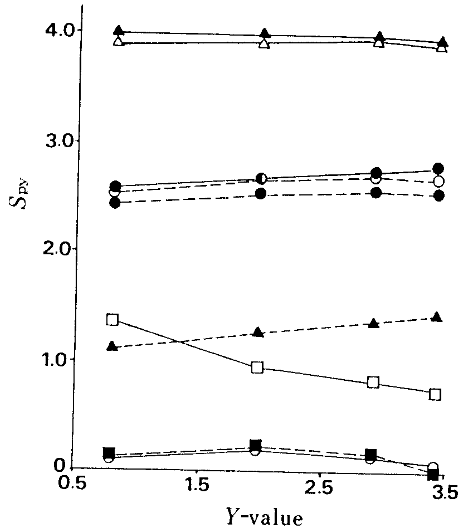

Fig. 2. Plots of S_{py} *versus* Y-Value of the Reaction Medium

MMS (—△—); EMS (---○---); iPMS (—□—); DMS (—▲—); DES (---●---); EtOTs (—●—); MNU (---▲---); ENU (—○—); iPNU (---■---).

TABLE II. Dependence of S_{py} on the pH of the Reaction Medium at 37 °C

Compound	Acetone content	S_{py} pH 6.0	S_{py} pH 7.4	ΔS_{py} [a]
MMS	4	3.86	3.72	0.14
	20	3.93	3.61	0.32
	40	3.87	3.55	0.32
	60	3.86	3.63	0.18
DMS	4	3.91	3.69	0.22
	20	3.95	3.71	0.24
	40	3.95	3.63	0.32
	60	3.96	3.62	0.34
DES	4	2.53	2.48	0.05
	20	2.54	2.46	0.05
	40	2.51	2.45	0.06
	60	2.41	2.42	−0.01
MNU	4	1.43	1.38	0.05
	20	1.37	1.30	0.07
	40	1.26	1.13	0.13
	60	1.11	1.03	0.08
ENU	4	2.66	2.64	0.02
	20	2.67	2.62	0.05
	40	2.64	2.56	0.08
	60	2.51	2.44	0.07

[a] $\Delta S_{py} = S_{py}(\text{pH } 6.0) - S_{py}(\text{pH } 7.4) = \log\{(k_{py}/k_{aq})_{6.0}/(k_{py}/k_{aq})_{7.4}\} = \log\{k_{aq}(\text{pH } 7.4)/k_{aq}(\text{pH } 6.0)\}$

than k_{py} by the solvent polarity.

Dependence of S_{py} on the pH of the Reaction Medium

The S_{py} values of some alkylating agents were measured in phosphate buffers at pH 6.0 and 7.4, and the results are shown in Table II. Provided that the rate of reaction with pyridine does not depend much on such a pH change, then the logarithm of the ratio of k_{py}/k_{aq} at pH 6.0 to that at pH 7.4, ΔS_{py}, approximates to that of the hydrolyses, $\log\{k_{aq}(\text{pH } 7.4)/k_{aq}(\text{pH } 6.0)\}$. As can be seen in the table, although $[OH^-]$ is $10^{1.4}$ ($=25$) times more concentrated in the pH 7.4 medium, the ΔS_{py} values observed are comparatively small; less than 0.1 in most cases and about 0.3 in typical S_N2 reactions of MMS and DMS. These results may indicate that hydrolysis takes place mainly through the reaction with H_2O molecule, especially in the

TABLE III. Dependence of S_{py} on Reaction Temperature

Compound	Acetone content	S_{py} at 37 °C	S_{py} at 60 °C	ΔS_{py}[a]
MMS	4	3.86	3.77	−0.09
	20	3.93	3.69	−0.24
	40	3.87	3.52	−0.35
	60	3.86	3.50	−0.36
EMS	4	2.66	2.46	−0.17
	20	2.67	2.50	−0.17
	40	2.64	2.45	−0.19
	60	2.51	2.39	−0.12
iPMS	4	0.72	0.67	−0.05
	20	0.83	0.76	−0.07
	40	0.95	0.85	−0.10
	60	1.35	1.32	−0.03
DMS	4	3.91	3.89	−0.02
	20	3.95	3.80	−0.15
	40	3.95	3.64	−0.31
	60	3.96	3.51	−0.45
DES	4	2.53	2.42	−0.11
	20	2.54	2.41	−0.13
	40	2.51	2.37	−0.14
	60	2.41	2.30	−0.11
EtOTs	4	2.76	2.63	−0.13
	20	2.72	2.56	−0.16
	40	2.66	2.52	−0.14
	60	2.56	2.44	−0.12
MNU	4	1.43	1.35	−0.08
	20	1.37	1.29	−0.08
	40	1.26	1.19	−0.07
	60	1.11	1.05	−0.06
ENU	4	0.07	0.07	±0.00
	20	0.15	0.14	−0.01
	40	0.21	0.19	−0.02

a) $\Delta S_{py} = S_{py}(60\,^{\circ}\mathrm{C}) - S_{py}(37\,^{\circ}\mathrm{C}) = \log\{(k_{py}/k_{aq})_{60\,^{\circ}\mathrm{C}}/(k_{py}/k_{aq})_{37\,^{\circ}\mathrm{C}}\}$

pH 6.0 medium. Neglecting for the moment the physical meaning of S_{py}, provided that one takes a fixed pH and ionic strength of the buffer for the analysis, one can evaluate the chemoselectivity by normalizing the product ratio with the molarities of water and pyridine, even if k_{obs} for hydrolysis involves, to an appreciable extent, the terms of $k_{OH}[OH^-]$ and k_{ph}[Phosphate].

Temperature Dependence of S_{py}

A preliminary study was made on the temperature dependence of the chemoselectivity.[14] Thus, the S_{py} values were measured at 60 °C and compared with those obtained at 37 °C, the results are shown in Table III. In all the cases examined, S_{py} tended to decrease with increase in the reaction temperature. This trend is also found in the data reported by Osterman-Golkar *et al.*[4] The energy of activation for pyridine quaternization may, in general, be slightly smaller than that for the hydrolysis in this series of reactions.

Discussion

The present report describes a simple analytical method to evaluate the relative chemoselectivity of alkylating agents. On the basis of the linear free energy relation in the kinetics, S_{py} might be written as follows.

$$\begin{aligned} S_{py} &= \log(k_{py}/k_{aq}) \\ &= sn_{py} \end{aligned}$$

where s is the substrate constant and n_{py} is the nucleophilicity constant of pyridine in the buffered medium employed. However, the correlated equation experimentally computed includes a constant term in the linear equation, as can be seen in Table II. One possible reason for the inconsistency may be the approximation involved in the present procedure; the rate constant for hydrolysis (k_{aq}) is replaced simply by k_w [H_2O]. Another possibility relates to the fact that s is the average value from reactions with a variety of nucleophiles under non-specified reaction conditions, whereas S_{py} is the relative rate ratio between the specified reactions with pyridine and aqueous oxygens under the specified reaction condition described here. Although the theoretical basis is obscure, it is evident that the S_{py} value can be a measure of the relative chemoselectivity, which is satisfactorily convertible to the substrate constant by the experimentally derived correlation equations.

Although the term "substrate constant" is generally used for the s value, its physical meaning is the chemoselectivity of electrophiles including alkylating agents. Chemoselectivity is definitely an important characteristic, in addition to the reaction rate, in determining what kind of and how much chemical injury is produced in biological molecules leading to biological alterations. Therefore, the evaluated constant for chemoselectivity may be introduced as a reaction parameter, in addition to Hammett's sigma value, in the correlation equation for quantitative structure–activity relationship (QSAR).

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