Influence of Serum Albumins on Decomposition Rates of *para*-Substituted 1-Phenyl-3-methyltriazenes and 5-(3-Methyl-1-triazeno)imidazole-4-carboxamide in Near Physiological Conditions

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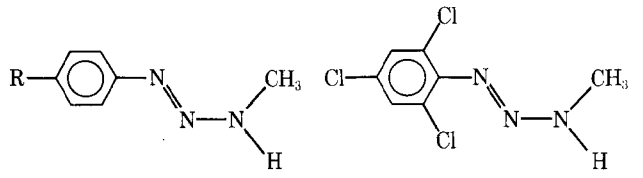
Abstract □ The influence of human serum albumin on the decomposition rates of some arylmonomethyltriazenes in buffered aqueous solution was investigated. From the experimental data, a model for the triazene–albumin interaction was derived, and the thermodynamic parameters were systematically calculated by two independent methods. The results show marked dependence of the energetics of binding on the substituent in the triazene aromatic ring. For most of the triazenes studied, the binding with albumin was mainly enthalpy driven. Measurements also were performed using bovine and murine serum albumins.

Keyphrases □ Arylmonomethyltriazenes—decomposition rates, effect of human serum albumin □ Serum albumin, human—effect on decomposition rates of arylmonomethyltriazenes □ Binding kinetics—triazene–albumin interaction, decomposition rates □ Antitumor agents—arylmonomethyltriazenes, effect of human serum albumin on decomposition rates

Aryldimethyltriazenes are a class of compounds possessing antitumor (1–4), carcinogenic (5–7), and mutagenic (8–10) activities. Evidence exists that metabolites of these triazenes are responsible for their *in vivo* effects. Monomethyltriazenes, produced by oxidative *N*-demethylation of the parent dimethyl compounds, are considered as possible metabolic species active at a systemic level (2, 11, 12) since they can alkylate cellular components by hydrolysis (13). Arylmonomethyltriazenes decompose in aqueous solution buffered at near neutral pH values to the corresponding anilines, nitrogen, and carbon cation derivatives with pseudo-first-order kinetics (14).

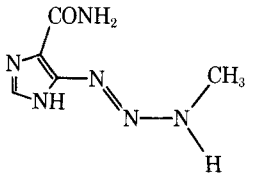
Since the possibility of interaction between these drugs and serum components exists, the influence of serum albumins on the decomposition rates of some *para*-substituted 1-phenyl-3-methyltriazenes, 1-(2,4,6-trichloro)-phenyl-3-methyltriazene, and 5-(3-methyl-1-triazeno)-imidazole-4-carboxamide was studied. The last compound is possibly a metabolite of 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (NSC 45388), used in the treatment of malignant melanoma. Decomposition kinetic studies of these triazenes also were performed in the absence of serum albumins, because the reported data show a marked dependence of the decomposition rates of arylmonomethyltriazenes on the medium (15) and because accurate data in near physiological conditions are lacking.

Human serum albumin was systematically employed. Bovine and mouse serum albumins also were used for comparison and, in the latter case, to allow correlations between the present results and possible *in vivo* experiments.



I–X

XI



XII

EXPERIMENTAL

Chemistry—The monomethyltriazenes (Table I) were synthesized by standard methods, *i.e.*, by coupling aryldiazonium chloride to two equivalents of methylamine in the presence of excess base, usually sodium carbonate. The coupling reactions, with the exceptions of VII and XI, were performed in the presence of large volumes of ether to extract the reaction products. To obtain very pure samples of I–VI and VIII, careful sublimation of small amounts of the raw materials, below their melting points, at ~0.2 torr was necessary. A chilling bath of dry ice–acetone was used to recover the recrystallized products; VII was purified by extracting the dried raw material with boiling ethyl acetate.

Purity of the monomethyltriazenes and the aromatic amines used as reference compounds was checked by NMR spectroscopy, elemental analysis, and TLC. Aluminum oxide and methanol–ethyl acetate–ligroin (3:2:1) or ethyl acetate–hexane (1:1) were used for TLC of monomethyltriazenes. The melting points were determined in open glass capillaries in a Büchi apparatus and were in accordance with the literature values. The references for the melting points are listed in Table I.

The solvents used for crystallizations were petroleum ether (bp 30–50°) for I, *n*-hexane for II–VI, ethyl acetate for VII, methanol for VIII, and benzene for IX.

Materials—The solvents used for TLC and crystallizations, as well as potassium dihydrogen *o*-phosphate and potassium hydroxide used in the preparation of buffer solutions, were reagent grade.

Serum albumins were crystallized and lyophilized human albumin, crystallized and lyophilized bovine serum albumin, mouse serum albumin fraction V powder, and crystallized and lyophilized egg albumin.

All serum albumins were used without further purification. Human and bovine serum albumin concentrations were determined spectrophotometrically using $E^{1\%}_{279} = 5.3$ and 6.6, respectively (22). Murine serum and egg albumin concentrations were determined by accurately weighing dried samples. Freshly prepared albumin, triazene, and buffer solutions were used.

Methods—All kinetic measurements were performed in 0.065 *M* phosphate buffer prepared using fresh, double-distilled water, specific conductivity $1.0 \times 10^{-6}\ \Omega^{-1}$/cm, adjusted to pH 7.40 at 25° by the addition of potassium hydroxide. The temperature dependence of pH of phosphate buffer solutions between 20 and 45° resulted in 2×10^{-3} pH unit per degree. This temperature dependence was taken into account; *i.e.*, all results of the kinetic measurements performed at different tem-

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Table I—Kinetic Data of the Pseudo-First-Order Decomposition of Monomethyltriazenes in Buffered Aqueous Solution [a]

Compound	R	Melting-Point Reference	T, °K	pH	$k \times 10^3$, sec^{-1}
I	H	17	294.9	7.405	4.78
			301.2	7.391	6.80
			310.8	7.371	11.3
			318.6	7.354	16.6
II	CH_3	18	293.5	7.408	7.76
			303.0	7.388	13.1
			310.2	6.965	49.8
			310.2	7.179	32.5
			310.2	7.372	18.0
			310.2	7.711	9.50
			310.2	7.984	4.92
			310.7	7.371	20.2
			318.7	7.354	28.6
III	F	16	296.2	7.402	3.33
			299.7	7.395	4.03
			304.2	7.385	5.47
			310.0	7.372	7.61
			311.1	7.370	8.68
			314.8	7.362	11.05
IV	Cl	19	295.2	7.405	1.71
			303.2	7.387	2.79
			310.2	7.372	4.27
			318.3	7.354	6.62
V	Br	16	296.7	7.401	1.55
			303.2	7.387	2.39
			310.2	6.965	9.67
			310.2	7.192	6.00
			310.2	7.372	3.63
			310.2	7.711	1.80
			310.2	7.984	0.878
			316.2	7.359	5.36
VI	I	19	296.1	7.403	1.57
			302.3	7.389	2.32
			308.8	7.375	3.32
			313.7	7.364	4.41
			314.5	7.363	4.64
VII	$CONH_2$	20	294.2	7.407	0.496
			303.2	7.387	0.965
			310.0	7.372	1.53
			317.9	7.355	2.48
VIII	$COOCH_3$	19	294.2	7.407	0.383
			296.9	7.401	0.439
			302.2	7.389	0.692
			304.2	7.385	0.742
			310.2	7.372	1.11
			311.1	7.370	1.23
			315.4	7.361	1.65
			318.2	7.355	1.70
IX	SO_2CH_3	2	302.9	7.388	0.318
			310.2	6.965	1.18
			310.2	7.192	0.824
			310.2	7.372	0.601
			310.2	7.711	0.351
			318.2	7.355	1.26
X	NO_2	20	294.2	7.407	0.272
			295.3	7.007	0.468
			295.3	7.369	0.319
			295.3	7.917	0.241
			302.2	7.389	0.666
			303.9	6.986	1.18
			303.9	7.160	1.07
			303.9	7.348	0.879
			303.9	7.692	0.711
			303.9	7.896	0.715
			310.2	6.965	2.42
			310.2	7.192	1.98
			310.2	7.372	1.73
			310.2	7.711	1.69
			310.2	7.984	1.38
			318.2	7.355	3.50
XI [b]			296.7	7.401	11.7
			298.2	7.398	13.6
			302.7	7.388	23.4
			307.4	7.378	36.8
			312.4	7.367	55.0
			314.3	7.363	74.5
XII		21	293.7	7.408	0.775
			302.2	7.389	2.31
			310.2	6.965	6.11
			310.2	7.192	5.89
			310.2	7.377	5.59
			310.2	7.984	5.36
			318.2	7.355	14.2

[a] Phosphate buffer, 0.065 *M*; ionic strength, 0.15 *M*. [b] This compound was a gift of Dr. G. F. Kolar, Institute for Toxicology and Chemotherapy, German Cancer Research Center, Heidelberg, West Germany.

peratures and pH values were interpolated at the common value of pH 7.40, using kinetic data determined as a function of pH between 7 and 8 for each triazene.

The decomposition reactions were followed spectrophotometrically at a fixed wavelength in the 310–350-nm range chosen to permit maximum variation of absorbance and minimum interference with the spectrum of the decomposition products. In each case, the spectrum at the end of the reaction was coincident with that of the corresponding aniline.

A freshly prepared solution ($\sim 4 \times 10^{-3}$ *M*) of monomethyltriazene in absolute ethanol was added to a known volume of aqueous phosphate buffer, in a quartz cell thermostated at ±0.05°, so that the ethanol–buffer volume ratio was 1:100 in all cases. The solution was stirred rapidly, and the absorbance was recorded as a function of time. The logarithm of the difference between the absorbance at time t and at the final time was plotted *versus* t; from the straight line so obtained, the pseudo-first-order kinetic constant was calculated using the least-squares method.

At the wavelength used, serum albumins gave rise to very low scattering, which was constant with time and did not invalidate the measurements.

The treatment of experimental data was the same both in the absence and presence of albumin, even if the values of the molar absorptivities of free and bound triazene species (ϵ_F and ϵ_B, respectively) might be different. This event does not invalidate the assumption of proportionality between the normalized optical density and the total triazene concentration during the kinetic measurements. An excess of C_P (protein concentration) with respect to C_B (bound triazene concentration) ensures near proportionality between C_B and C_F (free triazene concentration) and, therefore, between the optical density and C_T (total triazene concentration):

$$\text{optical density} = \epsilon_B C_B + \epsilon_F C_F \qquad \text{(Eq. 1)}$$

and by use of Eqs. 5 and 6, it follows that:

$$\text{optical density} \propto (K_B C_P \epsilon_B + \epsilon_F) C_T = \text{constant } C_T \qquad \text{(Eq. 2)}$$

The possibility that the aniline produced by the decomposition reaction interferes with the triazene binding sites (*e.g.*, by competition) was tested. Some sequential kinetic measurements, performed in the same protein solution that allowed the aniline concentration to rise, gave constant half-lives, indicating that the effect of the aniline in this respect is negligible.

Linearization of data, when necessary, was performed by a computerized least-squares procedure.

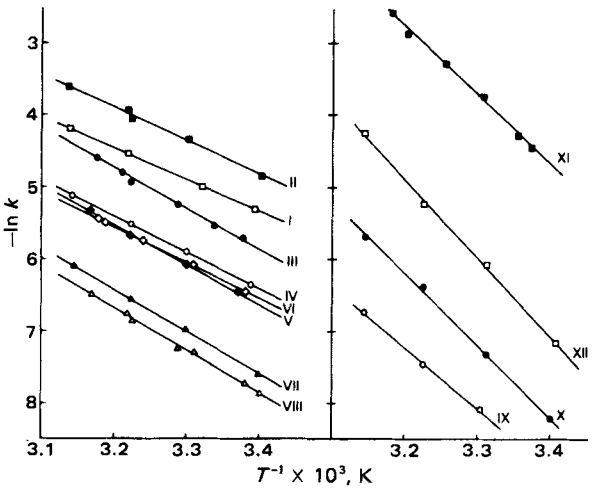

Figure 1—*Arrhenius plots for the monomethyltriazenes studied.*

Table II—Kinetic Data of the Pseudo-First-Order Decomposition of Monomethyltriazenes [a]

Compound	$k \times 10^3$, sec^{-1}	$t_{1/2}$, sec	ln A [b]	E, kJ × $mole^{-1}$ [b]	$C_P \times 10^4$, M	$k' \times 10^3$, sec^{-1}	$t'_{1/2}$, sec	ln A' [b]	E', kJ × $mole^{-1}$ [b]
I	10.2	68	9.8 ± 0.1	37.0 ± 0.1	1.05	8.6	81	12.2 ± 0.4	43.8 ± 1.1
II	17.7	39	10.0 ± 0.4	36.2 ± 1.1	1.20	11.5	60	13.1 ± 0.6	45.4 ± 1.6
III	7.56	92	12.9 ± 0.4	45.9 ± 1.1	0.98	6.36	109	13.2 ± 0.8	47.2 ± 2.1
IV	3.96	175	10.7 ± 0.1	41.9 ± 0.3	1.03	1.88	368	14.0 ± 0.3	52.4 ± 0.9
V	3.46	200	11.9 ± 0.2	45.3 ± 0.5	1.15	1.33	520	16.1 ± 1.8	58.9 ± 4.6
VI	3.40	204	10.3 ± 0.1	41.3 ± 0.4	1.00	0.99	698	19.1 ± 1.0	67.5 ± 2.5
VII	1.43	485	12.3 ± 0.2	48.7 ± 0.5	0.89	1.38	503	13.1 ± 0.1	50.6 ± 0.1
VIII	1.09	636	12.5 ± 0.4	49.8 ± 0.8	1.05	0.81	853	17.8 ± 0.2	64.1 ± 0.5
IX	0.589	1177	19.4 ± 0.6	69.3 ± 2.2	—	—	—	—	—
X	1.58	439	25.6 ± 0.7	82.7 ± 1.8	0.97	0.72	967	30.1 ± 2.0	96.6 ± 5.0
XI	47.6	15	27.6 ± 0.7	79.0 ± 1.7	1.00	11.6	60	17.7 ± 0.9	56.6 ± 2.3
XII	5.67	122	30.0 ± 0.6	90.8 ± 1.5	1.00	5.29	131	—	—

[a] The values of k, $t_{1/2}$, k', and $t'_{1/2}$ refer to 37° and pH 7.40. The prime denotes the presence of human serum albumin; C_P is the molar concentration of the protein. [b] Average ± SD.

RESULTS AND DISCUSSION

The kinetic law of decomposition of the monomethyltriazenes studied, carried out at various temperatures in pH 7.4 phosphate buffer (ionic strength 0.15 M), proved to be pseudo-first order with respect to the triazene concentration in all cases (Table I). Corrections for the slight temperature effect on pH and, therefore, on k were performed (see *Experimental*).

The Arrhenius plots of the kinetic data interpolated at pH 7.40 for all of the triazenes studied were fairly linear in the temperature range investigated (Fig. 1). By application of the empirical Arrhenius equation, $k = A \exp(-E/RT)$, to these kinetic measurements, the activation energies and the natural logarithm of the preexponential terms for the various triazenes were obtained (Table II).

The plot of log $k_{(R)}$ − log $k_{(H)}$ as a function of the Hammett constant σ (23, 24) for the different *para*-R-substituted aryl derivatives gives a satisfactory straight line, except for X and, to a lesser extent, for IX (Fig. 2). The slope of such a straight line is negative, in agreement with the expected electrophilic character of the decomposition reaction, with $\rho = -2$ approximately.

The triazenes that follow the Hammett plot are characterized by relatively low and approximately similar activation energies and preexponential terms (36–50 kJ $mole^{-1}$ and 10–13, respectively), whereas higher E and ln A values have been found for XI and XII and for the two triazenes (IX and X) that do not follow this plot (69–91 kJ $mole^{-1}$ and 19–30, respectively). The peculiar behavior of IX and X is likely to be attributed to the different extent of protonation of these triazenes at pH 7.4 as compared to the other compounds. This protonation would affect their behavior with respect to acid catalysis.

The results of the kinetic measurements show that the presence of human serum albumin in solution causes a more or less marked lessening in the decomposition rates of the triazenes considered, without affecting the order of the reaction (Fig. 3 and Table III). Therefore, the reaction rate can be written as:

$$v = -\frac{dC_T}{dt} = k'C_T \qquad \text{(Eq. 3)}$$

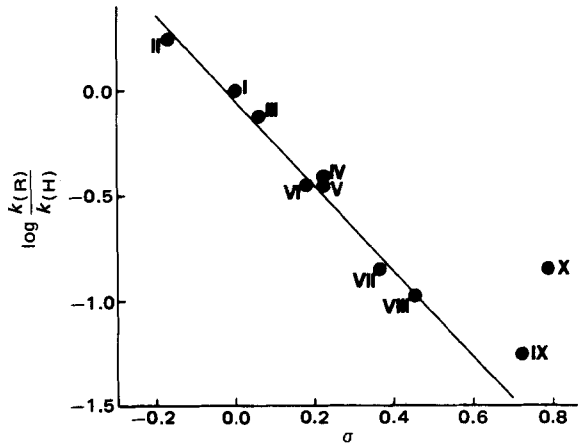

Figure 2—*Plot of* log $k_{(R)}$ −log $k_{(H)}$ versus *the Hammett constant,* σ, *for the various triazenes studied* ($\rho = -2$ *approximately).*

where k' is the apparent kinetic constant in the presence of albumin ($k' < k$).

The observed lessening in the decomposition rates caused by serum albumin may be interpreted by assuming that: (*a*) the triazenes interact rapidly and reversibly with a binding site onto the protein, (*b*) it cannot decompose when bound to this site, and (*c*) the fraction of unbound triazene decomposes in the bulk of the solution with kinetic constant k.

On the basis of the first assumption, the stoichiometric constant of association between the triazene and the protein, K_B, may be defined as:

$$K_B = C_B/C_F(C_P - C_B) \qquad \text{(Eq. 4)}$$

Since in the present case C_P is always greater than C_T and C_B generally is sufficiently smaller than C_T, C_B can be neglected with respect to C_P, and it is possible to approximate:

$$\frac{C_B}{C_F} = K_B C_P \qquad \text{(Eq. 5)}$$

and, therefore:

$$C_F = C_T/(1 + K_B C_P) \qquad \text{(Eq. 6)}$$

This approximation induces a slight underestimation of the K_B values, which is more relevant for the highest ones. This underestimation does

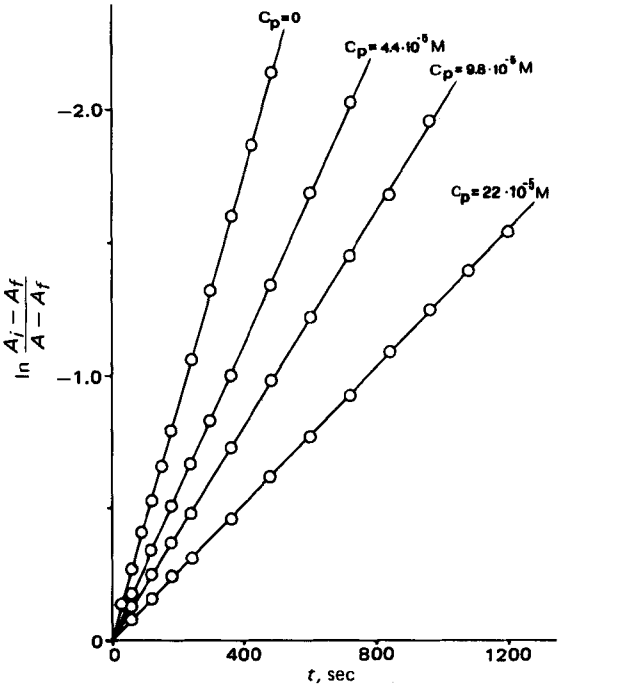

Figure 3—*Pseudo-first-order decomposition kinetics for 1-(*p*-chloro)-phenyl-3-methyltriazene at various human serum albumin concentrations at 37° and pH 7.40 (0.065 M potassium phosphate buffer);* A_i, A_f, *and* A *are the initial, final, and actual optical densities, respectively.*

Table III—Kinetic Data of the Pseudo-First-Order Decomposition of Monomethyltriazenes in Buffered Aqueous Solution [a] in the Presence of Human Serum Albumin

Compound	T, °K	$C_P \times 10^4$, M	pH	$k \times 10^3$, sec^{-1}
I	296.8	1.05	7.401	3.80
	302.9	1.05	7.388	5.73
	310.9	1.05	7.370	9.61
	317.7	1.05	7.356	13.4
	310.9	0.79	7.370	9.14
	310.9	1.59	7.370	8.29
	310.9	2.67	7.370	7.76
II	293.5	1.20	7.408	4.08
	302.7	1.20	7.388	7.20
	310.7	1.20	7.371	12.5
	310.2	0.70	7.372	13.3
	310.7	1.40	7.371	11.5
	311.2	2.80	7.370	8.47
III	299.7	0.98	7.395	3.14
	301.0	0.99	7.392	3.40
	305.6	0.98	7.382	5.01
	307.5	0.99	7.378	5.80
	311.1	0.98	7.370	6.83
	315.2	0.98	7.361	8.44
	310.3	0.49	7.372	7.26
	310.3	1.03	7.372	6.98
	310.3	2.05	7.372	5.50
IV	294.0	1.03	7.407	0.593
	303.0	1.03	7.387	1.20
	310.0	1.03	7.372	1.89
	318.0	1.03	7.355	3.43
	310.0	0.44	7.372	2.82
	310.0	0.98	7.372	2.03
	310.0	2.20	7.372	1.29
V	299.5	0.96	7.395	0.527
	303.4	1.15	7.387	0.854
	306.9	0.96	7.379	0.955
	310.2	1.10	7.372	1.47
	314.7	1.15	7.362	1.79
	310.2	0.55	7.372	1.90
	310.1	1.92	7.372	0.952
	310.2	2.20	7.372	0.982
VI	296.7	1.00	7.401	0.261
	302.7	1.00	7.388	0.424
	309.2	1.00	7.374	0.865
	313.9	1.00	7.364	1.22
	310.3	0.54	7.372	1.68
	310.3	1.08	7.372	0.960
	310.1	2.16	7.372	0.613
VII	294.0	0.89	7.407	0.468
	303.0	0.89	7.387	0.910
	318.0	0.89	7.355	2.52
	310.0	0.41	7.372	1.48
	310.0	1.50	7.372	1.41
	310.0	2.90	7.372	1.41
VIII	395.9	1.05	7.403	0.255
	303.7	1.05	7.386	0.532
	309.4	1.05	7.374	0.850
	316.0	1.05	7.359	1.49
	302.0	1.20	7.390	0.421
	318.0	1.20	7.355	1.21
	310.0	0.51	7.372	0.975
	310.0	1.20	7.372	0.844
	310.0	2.20	7.372	0.675
	310.0	4.00	7.372	0.505
X	294.0	0.97	7.407	0.091
	302.0	0.97	7.390	0.211
	318.0	0.97	7.355	1.77
	310.0	0.38	7.372	1.03
	310.0	0.86	7.372	0.831
	310.0	1.30	7.372	0.558
	310.0	2.40	7.372	0.425
XI	296.7	0.99	7.401	5.95
	297.9	1.00	7.399	5.42
	302.7	0.99	7.388	8.91
	307.4	1.00	7.378	11.5
	311.1	1.00	7.370	16.4
	316.8	1.00	7.358	23.4
	307.4	0.50	7.378	18.3
XII	310.0	1.20	7.372	5.32
	310.0	2.50	7.372	4.72

[a] Phosphate buffer, 0.065 M; ionic strength, 0.15 M.

not exceed 10% even in the less favorable case of the highest K_B values ($\sim 2.10^4\ M^{-1}$), being slightly greater than the standard deviation due to random errors. This evaluation was done by choosing the values of C_P and C_T in the middle of their concentration range ($\sim 1.5 \times 10^{-4}$ and $2 \times 10^{-5}\ M$, respectively).

A more rigorous treatment that avoids the approximation leading to Eq. 5 is given in the *Appendix*.

Following the previous assumptions, it is possible to state that:

$$v = -\frac{dC_T}{dt} = kC_F = kC_T/(1 + K_BC_P) \qquad \text{(Eq. 7)}$$

where v is the decomposition rate. Therefore:

$$k' = k/(1 + K_BC_P) \qquad \text{(Eq. 8)}$$

and:

$$t'_{1/2} = t_{1/2}(1 + K_BC_P) \qquad \text{(Eq. 9)}$$

where $t'_{1/2}$ and $t_{1/2}$ are half-life times of the triazenes in the presence and absence of protein, respectively. Equations 8 and 9 are justified in the assumption that $C_P > C_T$.

For the triazenes considered, a plot of $t'_{1/2}/t_{1/2}$ *versus* C_P gives a straight line with slope K_B (Fig. 4). These K_B values are reported in Table IV.

Another possible model, *i.e.*, a tight binding of the triazene onto the protein and the subsequent decomposition *in situ*, is ruled out since, in such a case, k' would be independent of C_P since C_P is always greater than C_T in the experiments.

The proposed theoretical scheme and the K_B values deduced were confirmed by a series of independent kinetic measurements carried out for each triazene at constant C_P and various temperatures between 20 and 45°.

Within the experimental accuracy (±3% approximately), the data also follow the Arrhenius equation in the presence of serum albumin, *i.e.*:

$$\ln k' = \ln A' - E'/RT \qquad \text{(Eq. 10)}$$

where the prime denotes the presence of albumin.

The activation parameters for all monomethyltriazenes studied are reported in Table II.

The introduction of the Arrhenius equation in Eq. 8 gives:

$$K_B = \frac{\frac{A}{A'}\exp\left(-\frac{E - E'}{RT}\right) - 1}{C_P} \qquad \text{(Eq. 11)}$$

Thus, Eq. 11 provides an independent way of obtaining K_B; the values so obtained are reported in Table IV. They are in agreement with those values determined from the measurements made as a function of C_P.

Moreover, from the temperature dependence of K_B, the binding enthalpies of the various triazene molecules onto the protein, ΔH_B, can be estimated using the derivation of Eq. 11:

$$\Delta H_B = -\frac{\partial R \ln K_B}{\partial\left(\frac{1}{T}\right)} = \frac{(E - E')\frac{A}{A'}\exp\left(-\frac{E - E'}{RT}\right)}{\frac{A}{A'}\exp\left(-\frac{E - E'}{RT}\right) - 1}$$

$$= (E - E')\left(1 + \frac{1}{K_BC_P}\right) \qquad \text{(Eq. 12)}$$

Thus, by using K_B, the free energy of binding, ΔG_B ($\Delta G_B = -RT \ln K_B$), and ΔH_B values, the variation of binding entropy for the various triazenes can be obtained. Thermodynamic data are reported in Table IV. By inspection of these data, it can be seen that most of the triazenes considered interact with human serum albumin, mainly by an enthalpy-driven process, the entropic contribution to the ΔG_B values generally being quite low. This effect is more pronounced in VII and VIII, for which a larger enthalpy loss is paralleled by a strikingly larger entropy loss. Different behavior characterizes the trichloro-substituted compound, which completely reverses the signs of ΔH_B and ΔS_B and reveals a mainly entropy-driven binding to albumin, possibly related to a large desolvation of the triazene molecule in this process.

The smallest K_B values are shown by triazenes VII and XII, the only compounds characterized by the presence of an amido group. Within the corresponding series of dimethyltriazenes, those carrying the carboxamide function are among the most active against experimental rodent tumors (4).

Kinetic measurements also were performed at constant temperature and in the presence of bovine and murine serum albumins. Although for the latter the quality of the measurements was limited by the low degree of purity of the available sample, in both cases the kinetics of reaction

Table IV—Thermodynamic Data of Binding of Monomethyltriazenes by Human Serum Albumin at 37° in Phosphate Buffer (pH 7.40, Ionic Strength 0.15 M) [a]

Compound	$K_B \times 10^{-3}$, M^{-1}		$-\Delta G_B$, kJ × mole^{-1}		$-\Delta H_B$, kJ × mole^{-1}	ΔS_B, J × mole^{-1} × °K^{-1}
	Measurement 1[b]	Measurement 2[c]	Measurement 1[b]	Measurement 2[c]		
I	1.86 ± 0.13	2.2 ± 0.3	19.4 ± 0.2	19.9 ± 0.4	42 ± 7	−70 ± 20
II	5.07 ± 0.06	4.9 ± 0.3	22.0 ± 0.1	21.9 ± 0.2	24 ± 5	−10 ± 20
III	1.94 ± 0.18	2.6 ± 0.3	19.5 ± 0.2	20.3 ± 0.3	8 ± 15	40 ± 50
IV	10.8 ± 0.2	11.1 ± 0.2	24.0 ± 0.1	24.0 ± 0.1	20 ± 2	10 ± 10
V	13.3 ± 0.5	15.4 ± 0.5	24.5 ± 0.1	24.9 ± 0.1	22 ± 8	10 ± 20
VI	22.4 ± 0.9	30.7 ± 1.1	25.8 ± 0.1	26.6 ± 0.1	38 ± 4	−40 ± 10
VII	0.34 ± 0.05	0.3 ± 0.1	15.0 ± 0.4	14.7 ± 0.9	65 ± 20	−160 ± 60
VIII	2.84 ± 0.05	2.6 ± 0.2	20.5 ± 0.1	20.3 ± 0.2	62 ± 4	−130 ± 10
X	13.4 ± 0.5	14.4 ± 1.1	24.5 ± 0.1	24.7 ± 0.2	25 ± 9	0 ± 30
XI	21.7 ± 0.4	22.1 ± 0.5	25.8 ± 0.1	25.8 ± 0.1	−34 ± 4	190 ± 10
XII	0.70 ± 0.07	—	16.9 ± 0.3	—	—	—

[a] Average ± SD. [b] From kinetic measurements at 310 °K and various albumin concentration values. [c] From kinetic measurements at constant albumin concentration and various temperatures.

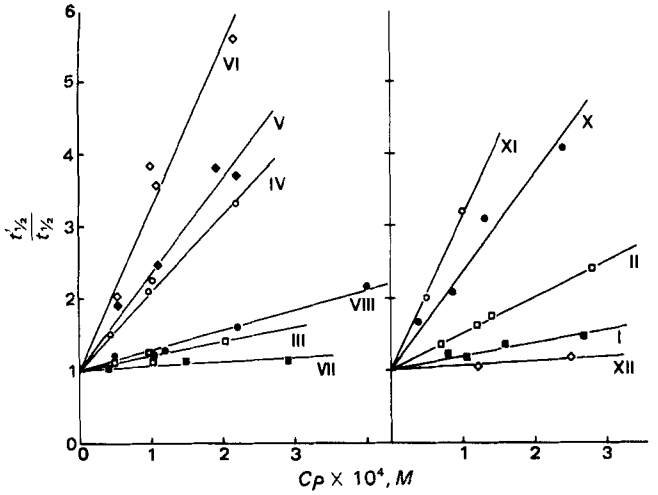

Figure 4—*Half-life times ratios, $t'_{1/2}/t_{1/2}$ (where the prime denotes the presence of human serum albumin) as a function of the protein concentration for the triazenes studied. The values refer to 37° and pH 7.40 (0.065 M potassium phosphate buffer).*

clearly remained pseudo-first order and the magnitude of the K_B values was markedly similar to that obtained with human serum albumin. The kinetic and binding parameters are reported in Table V.

However, some kinetic experiments carried out in the presence of egg albumin showed no appreciable effect of this protein on the decomposition rates of the triazenes. This result, obtained in the presence of a protein similar to the serum albumins in some physicochemical properties and in amino acid composition but different in its biological role, confirms that the effect of the serum albumins on the decomposition rates of the triazenes is due to their specific binding abilities.

This protein binding can greatly influence the triazene half-life times in comparison with the values obtained in protein-free solutions. This interaction has to be taken into account in the attempt to correlate kinetic data *in vitro* with biological activities *in vivo*, although the extent of this interaction cannot be predicted easily on the basis of structural or physicochemical parameters.

A preliminary attempt to check, at least qualitatively, the influence of other serum components on the decomposition reactions and binding was performed using whole horse serum. The serum was diluted with phosphate buffer to reach a serum albumin concentration comparable with concentrations used in the previous experiments. The reaction still was pseudo-first order, and the half-lives were definitely close to those obtained in the presence of pure serum albumin (Table V). This result seems to indicate that the major part of the observed effect of the serum components on the triazene decomposition rates is to be attributed to the albumin fraction.

APPENDIX

Exact solution of Eq. 4 for C_F, avoiding the simplifying condition $C_B \ll C_P$, leads to:

$$C_F = \tfrac{1}{2}\left[C_T - C_P - \frac{1}{K_B} + \sqrt{\left(C_P - C_T + \frac{1}{K_B}\right)^2 + \frac{4C_T}{K_B}}\right] \quad \text{(Eq. A1)}$$

The kinetic equation:

$$\frac{dC_T}{C_F} = \frac{dC_T}{\tfrac{1}{2}\left[C_T - C_P - \frac{1}{K_B} + \sqrt{\left(C_P - C_T + \frac{1}{K_B}\right)^2 + \frac{4C_T}{K_B}}\right]} = -k\,dt \quad \text{(Eq. A2)}$$

has to be integrated (from initial conditions: $C_T = C_{T,0}$ at $t = 0$).

This goal can be achieved considering that:

$$\frac{dC_T}{C_F} = \frac{dC_T}{dC_F}\frac{dC_F}{C_F} \quad \text{(Eq. A3)}$$

where:

$$\frac{dC_T}{dC_F} = \frac{C_F^2 + \frac{2C_F}{K_B} + \frac{1}{K_B}\left(C_P + \frac{1}{K_B}\right)}{\left(C_F + \frac{1}{K_B}\right)^2} \quad \text{(Eq. A4)}$$

so that:

$$\int_{C_{T,0}}^{C_T}\frac{dC_T}{C_F} = \int_{C_{F,0}}^{C_F}\left[\frac{C_F + \frac{2}{K_B} + \frac{1}{K_B C_F}\left(C_P + \frac{1}{K_B}\right)}{\left(C_F + \frac{1}{K_B}\right)^2}\right]dC_F = -k\int_0^t dt \quad \text{(Eq. A5)}$$

Table V—Kinetic and Thermodynamic Data of the Decomposition of Monomethyltriazenes in Buffered Aqueous Solution in the Presence of Different Serum Albumins (0.065 M Potassium Phosphate Buffer, pH 7.40, and 37°)

Compound	Protein-Free Solution, $t_{1/2}$, sec	Human Serum Albumin		Bovine Serum Albumin		Murine Serum Albumin		Horse Serum, $t_{1/2}$, sec[c]
		$t_{1/2}$, sec[a]	$K_B \times 10^{-3}$, M^{-1}	$t_{1/2}$, sec[a]	$K_B \times 10^{-3}$, M^{-1}	$t_{1/2}$, sec[b]	$K_B \times 10^{-3}$, M^{-1}	
II	39	70	5	85	8	85	8	100
IV	175	460	11	540	14	520	13	700
VII	485	510	0.3	500	0.2	490	0.1	700
X	439	1320	13	850	6	1050	10	1000
XII	122	135	0.7	145	1	120	0	130

[a] Values linearly interpolated at albumin concentration 1.5×10^{-4} M. [b] Experimental values at protein concentration 1.5×10^{-4} M. [c] Experimental values in whole horse serum diluted 1:4 with phosphate buffer.

Finally, the integrated function $F(C_F)$ results:

$$F(C_F) \equiv K_B C_P \left[\frac{1}{1 + K_B C_F} - \ln\left(C_F + \frac{1}{K_B}\right)\right] + (1 + K_B C_P) \ln C_F = F(C_{F,0}) - kt \quad \text{(Eq. A6)}$$

being obviously C_F expressed as a function of C_P by Eq. A1.

This formal treatment may easily be extended to the case of n identical and independent binding sites present on the protein, simply by substituting C_P by nC_P in these expressions.

The mathematical treatment would be more complicated if nonidentical and possibly cooperative sites were present. Owing to the good agreement between experimental data and the described model, this last case was not considered.

In principle, Eq. A6 implies that the kinetic law for the decomposition of triazenes in the presence of albumin is not first order with respect to triazene concentration. It is easy to realize, by numerical substitution, that the deviation from first-order kinetics is almost negligible in the present case.

For several triazenes, it has been possible to plot the experimental data obtained for each triazene at constant temperature and various C_P values in the form of the general function $F(C_F)$ *versus* t (Eq. A6). All slopes at various C_P values were matched successfully with the value for the kinetic constant, k, in the absence of protein by choosing the same K_B value for each triazene. The same data, plotted according to the simplified form of Eq. 9, provided K_B values in good agreement with those obtained by the more rigorous treatment.

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Extreme Vertexes Design in Formulation Development: Solubility of Butoconazole Nitrate in a Multicomponent System

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Abstract □ The extreme vertexes design was shown to be an efficient method for the study of mixture problems, for generating points in the factor space that define a region for response surface analysis. By using this method, the solubility of butoconazole nitrate, an imidazole antifungal agent, was studied as a function of four components, polyethylene glycol 400, glycerin, polysorbate 60, and water, whose levels were subject to given constraints. A fifth component, poloxamer 407, was held constant. The design was used to generate 14 points in the region defined by the constraints. The G efficiency of the design, with the assumption of a quadratic model for the response surface, was 79%. By using the solubilities determined at the 14 points and regression analysis, an equation was generated to characterize the response surface. Contour plots of the response surface illustrate the relationship of the solubility as a function of the components, and solubilities calculated at other points (in the region) agree well with the observed data.

Keyphrases □ Extreme vertexes design—formulation development, butoconazole nitrate solubility in a multicomponent system □ Formulation development—extreme vertexes design, butoconazole nitrate solubility in a multicomponent system □ Butoconazole nitrate—solubility, extreme vertexes design in formulation development □ Multicomponent systems—extreme vertexes design in formulation development, butoconazole nitrate solubility

A pharmaceutical formulation generally consists of a mixture of several components, whose levels may often be constrained by factors other than those directly determining its physical properties, such as irritation and cost. In developing this formulation, the component levels must be varied within these constraints to arrive at an optimum formulation with respect to several responses such as solubility, stability, and bioavailability. The conventional