Vol. 1, Pergamon Press, New York, N. Y., 1965, pp 320–329, 357–364.
(16) W. L. Lipschitz, Z. Hadidian, and A. Kerpcsar, *J. Pharm. Exp. Ther.*, 79, 97 (1943).
(17) J. E. Leffler and E. Grunwald, "Rates and Equilibrium of Organic Reactions," Wiley, New York, N. Y., 1963, p 173.
(18) Y. H. Wu, W. G. Lobeck, Jr., and J. W. Kissel, *J. Med. Chem.*, 13, 975 (1970).

Receptor Site Labeling Through Functional Groups. 2. Reactivity of Maleimide Groups[1]

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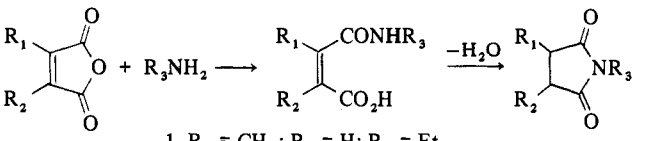
N-Alkylmaleimides are reactive groups suitable for binding drug fragments (D) linked to them *via* a chain (C_n) to receptor sites which bear suitable functional groups (*e.g.*, thiol groups). To evaluate the theiotropic (thiol-seeking) character of maleimides, rates of reaction with glutathione (GSH) in aq buffer have been examined. GSH adds to *N*-ethyl-2-methylmaleimide at the same atom as that which carries the Me group, demonstrating that electronic effects are far more important than steric effects in this reaction. Addition of GSH to *N*-ethyl-2,3-dimethylmaleimide reaches equilibrium in a solution containing 90–95% adduct. Based on the kinetic results, several 2-methylmaleimidylbarbiturate derivatives have been prepared and tested; modest signs of biological activity appeared for one compound. The rate constants permit rational choice of appropriate functional groups, provide insight into the biological effects of *N*-alkyl (or aryl-)-2,3-dichloromaleimides, and lead to a logical prediction for molecular modification of the maleimide antibiotic, showdomycin.

We have described an approach to the labeling of receptor sites, having a suitable functional group in proximity to the site, through the synthesis of molecules incorporating a drug fragment, D, a connection, C_n, and a reactive moiety, Y. The Y moiety was chosen for its theiotropic (thiol-seeking) properties and in our first effort, limited to two maleimidyl groups of extremely different reactivities.[1,‡]

In order to provide a graded range of Y reactivity, we have now evaluated a number of additional N-substituted maleimides, with respect to the rate of reaction with the tripeptide thiol, glutathione (GSH), in aq buffer. On the basis of the rate constants for the reaction between maleimides and GSH, several barbiturate derivatives were selected and synthesized.

Results

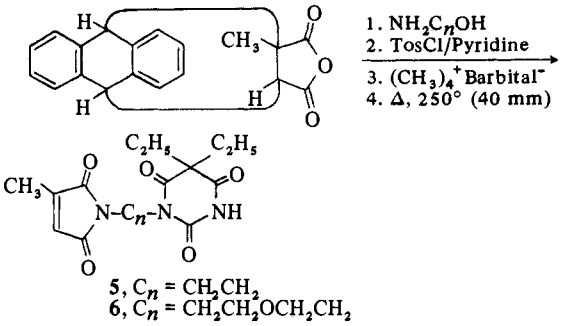
Synthesis. N-Substituted maleimides are prepared from the corresponding maleic anhydride *via* the intermediate maleamic acids (eq 1).



1, $R_1 = CH_3$; $R_2 = H$; $R_3 = Et$
2, $R_1 = CH_3$; $R_2 = CH_3$; $R_3 = Et$
2H, $R_1 = CH_3$; $R_2 = CH_3$; $R_3 = HOCH_2CH_2$
3, R_1–$R_2 = (CH_2)_4$
4, R_1–R_2 = 

(1)

Two barbiturate derivatives of *N*-ethyl-2-methylmaleimide (**1**), with either CH_2CH_2 (**5**) or $(CH_2)_2O(CH_2)_2$ (**6**) connecting the drug fragment to reactive group, were synthesized by the general route used previously.[1] The additional steric hindrance introduced by the 2-Me group necessitated the use of anthracene as a protecting group for the double bond of the maleic acid anhydride. A summary equation indicated the pathway (eq 2).



(2)

5, $C_n = CH_2CH_2$
6, $C_n = CH_2CH_2OCH_2CH_2$

Kinetic Studies. The rate of the disappearance of uv absorption due to the *N*-ethylmaleimide or maleimide was followed after dissolving GSH in a deoxygenated solution of maleimide in aq buffer. Strict second-order kinetics were followed in all cases except that of the 2,3-dimethylmaleimide which followed a rate equation for a bimolecular reaction to an equilibrium between products and reactants. The rate constants derived from the studies of the kinetics of the reaction are listed in Table I.

Biological Tests. According to the idea that maleimides function *via* reaction with SH groups within biological systems (either GSH or protein SH groups, with the former reacting much faster), maleimides should vary in biological effect in a fashion parallel to their rates of reaction with GSH. Indeed, by ip injection, NEM (**7**) was much more toxic than NEC (**1**) which, in turn, was far more toxic than DEM (**2**). A barbiturate linked to NEC by a C_2 chain (**5**) was similar to NEC in toxicity. At usual barbiturate levels, one linked through a C_2OC_2 chain to NEC (**6**) (*i.e.*, **202C**) was considerably more toxic, indicating that the depressant action of the barbiturate may have added to the toxic character of the NEC portion of the molecule. At low dose levels, the biological effects produced by **202C** were definitely more marked than those produced by the same levels of NEC. The biological activity of **6** is consistent with the activity found for a C_2OC_2-linked barbiturate derivative of dimethylmaleimide.[1]

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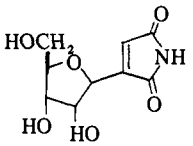
‡The term "thiophilic" refers to nucleophilicity *toward* sulfur[2] whereas "theiotropic" activity depends on the nucleophilic activity *of* sulfur as a thiol.

Table I. Rate Constants for the Reaction of N-Substituted Maleimides with Glutathione in Aqueous Buffer at 25°[a]

Maleimide	pH[b]	λ, nm	k, M^{-1} sec^{-1}	Relative k
N-Et (7)[c]	(7.3)[c]		2300	3.15×10^5
2-Me-*N*-Et[d] (1)	7.2	300.0	4.69	640
2,3-Tetramethylene-*N*-Et (3)	7.1	307.5	0.0295	4
2,3-(1,3-Cyclopentylene)-*N*-Et[e] (4)	7.2	325.0	290	4×10^4
2,3-Me_2-*N*-Et (2)	7.2	305.0	0.0073	1
Showdomycin (2-β-D-ribofuranosyl) (8)	7.2	280.0	295	4×10^4
				
2-Methyl (9)	7.2	270.0	5.17	710

[a]Measured in the absence of O_2 as described in the Experimental Section. [b]For the reaction mixt after the kinetic measurements. [c]Estimated for pH 7.3 from kinetic data reported at lower pH.[3] [d]Stable in buffer soln (7% change in absorbance over 7 days). [e]Absorption due to compd disappears at a moderate rate in aq buffer. The GSH reaction is carried out at concns for which the GSH reaction is the only significant one.

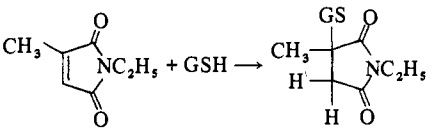
Table II. Biological Effects of *N*-Ethylmaleimides[a]

Compd	Dose, μmoles/animal[c]	Route[b]	Comment
NEM(7)	80	Ip	Death within 40 min
	40	Ip	Death within 120–150 min
	20	Ip	Death within 240–300 min
	2.0	Ic	Death within 10–60 min[d]
NEC(1)	80	Ip	Death within 4–12 hr
	40	Ip	Death within 5–20 hr
	20	Ip	Weakness, slow recovery over 24 hr
	9	Ic	Death within 6–8 hr
	5	Ic	No apparent effect after transient weakness
DEM (2)	80	Ip	Slight transient weakness in 50% of animals over 1–3 hr
	6.0	Ic	Transient cyanosis and weakness[c]
THE (3)	60	Ic	No observable effect[e]
2C (5)	60	Ip	Death in 10–20 hr
	40	Ip	Weakness for a few hr
202C (6)	80	Ip	Death 80–120 min
	10	Ip	Severe weakness (3–4 hr) recovery within 16 hr
	5	Ip	Groggy (1 hr), recovery after 2–3 hr

[a]The authors are grateful to Professor N. S. Kosower, Albert Einstein College of Medicine, for carrying out these experiments. [b]Ip = intraperitoneal; Ic = intracerebral. [c]Animals: Sprague–Dawley female rats averaging 150 g in wt were used. 2–5 animals for each dose level. [d]Lowest dose which produced 100% mortality. [e]As reported in Table VI, ref 1.

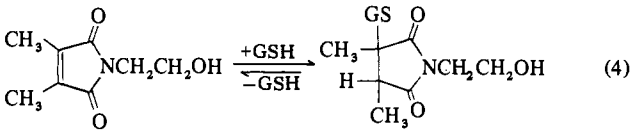
The tests, summarized in Table II, confirm the notion that biological activity can be controlled through structural variations in the maleimide portion of a maleimide derivative.

Products of Reaction of GSH with Maleimides. Nmr spectra of reaction mixtures of GSH and the 2-methylmaleimide (1) revealed quite clearly that the thiol added to the double bond at the same carbon as that to which the Me was bonded. The doublet due to the Me on the double bond disappeared after the reaction and was replaced by a *singlet* at much higher field than that at which the original Me doublet was observed in CCl_4. Although 1 was not very soluble in H_2O, a suspension of 1 quickly cleared after GSH was added (eq 3).



(3)

N-Ethyl-2,3-dimethylmaleimide (2) is not soluble enough in H_2O to allow the carrying out of an nmr experiment. The corresponding *N*-2-hydroxyethyl derivative (2H) was synthesized[1] and used in its place. At pH 7, equilibrium was reached about 5 hr after *ca.* 0.6 *M* solns were mixed at about 90% completion, using the ratio of the Me signals of the adduct to those of the maleimide to estimate the composition of the soln (eq 4).

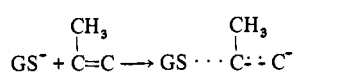


(4)

Discussion

To design labels for receptor sites, one must first acquire reasonably precise information on the activity of the reactive groups used to bind D-C_n-Y to the functional groups at or near the site. We have selected maleimides as a potentially general Y group, one with well-established theiotropic properties. Since the tripeptide thiol, glutathione (GSH), is one of the major[1,4] biological barriers to free distribution of molecules bearing a theiotropic group within a particular biological system, it is necessary to determine the reactivity of maleimides of interest with GSH. The results obtained thus far suggest that the steric requirements of the transition state for addition of GSH to the double bond of the maleimide are not severe, and the relative rate constants for the reactions of GSH with maleimides may thus reflect the relative reactivities of maleimides toward protein thiol groups.

Rate Patterns. A single Me group on the maleimide double bond reduces the reactivity toward GSH by a factor of about 500. (Compare k(7) with k(1), Table I.) A second Me group diminishes the reactivity toward GSH a further factor of 650 (k(1) and k(2), Table I). The only observable product of addition of GSH to the monomethylmaleimide (1) is that in which the thiol group has added at the position bearing the Me group. Thus, of the transition states illustrated in eq 5 and 6, that shown in eq 5 is at least 2 kcal/



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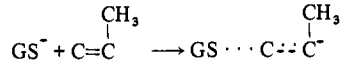


(6)

mole lower in energy than that shown in eq 6. It is clear that the transition state resembles the initially formed anion since we would expect the methyl group to diminish markedly the capacity of the carbon to carry its share of the negative charge, perhaps by 1–2 kcal/mole (for discussion of acidities *cf.* ref 5). We can thus understand the formation of the adduct at the substituted position, although we do not know precisely how much difference there is between the transition state to the observed product and the transition state to the product which was *not* observed, that product arising from addition at the unsubstituted position. We can infer from the fact that the transition state in eq 5

is favored that steric effects are not important in determining the course of the reaction.

Given the nature of the probable transition state, we would not expect a significant rate change from unsubstituted to methyl substituted because the methyl group is not in a position to influence the charge distribution greatly. To explain the rate change, we look at the transition energy differences corresponding to the rate factors. For k_7/k_1 and k_1/k_2, the $\Delta\Delta F^*$ values are 3.6 and 3.8 kcal/mole, respectively. Correcting for the fact that 7 and 2 have two equivalent positions, the $\Delta\Delta F^*$ values are 3.3 and 4.1 kcal/mole, respectively. The $\Delta\Delta F^*$ of 3.3 kcal/mole is very close to the 2.8 kcal/mole Me group stabilization of a double bond as measured by heats of hydrogenation.[6] Thus, the major part of the difference in rate constants observed for successive Me substitutions is due to Me stabilization of the initial state.

These considerations permit us to understand the rate and product effects of substitution into the maleimide, and can be extended, as the following example shows.

In contrast to the tetramethylene maleimide (**3**) with a reactivity only a little higher than that of the 2,3-dimethyl compound, the bicyclo[2.2.1]heptane-2 analog (**4**) is 40,000 times as reactive as the dimethyl derivative. The transition state energy difference, 6.3 kcal/mole, is a little higher than might have been expected for the increase in the initial state energy (= strain energy), 4.8 kcal/mole, based on heat of hydrogenation[7] for bicyclo[2.2.1]heptane. Perhaps fusing a 5 ring onto the system leads to a further increase in strain energy.

The antibiotic showdomycin (**8**) represents the only member thus far of the new class of maleimide nucleosides.[8] Its reactivity toward GSH was therefore of great interest. Showdomycin, a monosubstituted maleimide, was 63 times as reactive as the monomethylmaleimide **1** toward GSH. Replacement of the *N*-Et (as in **1**) by H (as in **9**) increases the rate of SH addition by only a factor of 1.1. Factors of 50–200 have been noted for the replacement of *N,N*-dimethylamide by amide in diazene derivatives, with respect to reaction with GSH.[9] The difference between the reactivity of **8** and that of **1** can be accounted for by the idea that the ether O of the ribofuranoyl ring would increase the ability of the maleimide system to accomodate negative charge. Some intramolecular H bonding in the transition state may also contribute to the reactivity of **8**.

We can conclude that maleimide derivatives will exhibit a very wide range of rates of reaction with GSH, and it is certainly reasonable to expect that the reactivity of a particular type of maleimide derivative can be predicted fairly well. It is useful to note that the position of the longest wavelength light absorption is fairly sensitive to the substituents on the maleimide (Table III).

Molecular Modification of Showdomycin. The mechanism of the biological action of showdomycin is not established but is generally agreed to involve thiols within the cell or in the membrane. A variety of effects have been observed including abnormal RNA synthesis and inhibition of growth.[8] Showdomycin resistance has been shown to correlate with abnormal ribosomal RNA and protein compositions[10] suggesting a substantial intracellular action for the antibiotic. Others suggest that transport[11] is the primary target for showdomycin.

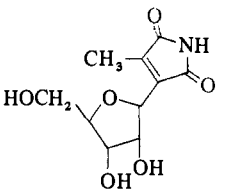
Table III. Position of Longest Wavelength Light Absorption for Substituted Maleimides[a]

Compd	λ_{max}, nm	ϵ_{max}
7	294.0	570[b]
1	297.0	255
2	305.0	190
3	310.0	170
4	320.0	122
8	290.0 sh[c]	601
9	272.0	273

[a] Spectra were measured in aq 0.5 *M* phosphate buffer, pH 7.2. [b] Measured in MeOH. [c] Shoulder.

The rate of the reaction of **8** with GSH is probably too high to allow complete distribution of the substance throughout a target organism of any complexity. A substance with comparable reactivity toward GSH, diamide,[4,12] affects only the GSH in red blood cells after iv administration to rabbits.§ Accepting the suggestion that showdomycin resembles a uridine nucleoside,[13] models suggest that there might be room for a Me group at the 3 position of the maleimide ring in combinations which are supposed to take the place of uridine nucleosides. The Me group might even enhance binding in a locally hydrophobic region. Most important, the Me group should diminish the rate for the reaction of the maleimide with GSH by a factor of about 500, bringing the rate constant down to 1 M^{-1} sec^{-1} or less.

These considerations suggest that 2-methyl-3-β-D-ribofuranosylmaleimide (**10**) should be a more effective antibiotic than showdomycin (**8**). The synthesis of **8** has been accomplished;[14] a shorter route would be desirable for the synthesis of **10**.



10

Other Maleimides. N-Substituted 2,3-dichloromaleimides have been utilized as fungicides and insecticides, and are apparently of some interest for other possible biological activities.[15-20]

At neutral pH, the reaction of *N*-ethyl-2,3-dichloromaleimide with GSH is extremely fast with a rate constant somewhat lower than that for NEM (**7**).# A serious question is therefore raised about the nature of the biologically active species present when 2,3-dichloromaleimides are applied to biological material.

Comparison of the rate results with those for GSH with other[21-23] alkenes substituted by electron-withdrawing substituents shows that maleimides are highly reactive theiotropic groups. The investigations of Anderson and Vasini on the inhibition of papain with *N*-alkylmaleimides, in which the length of the alkyl chain is varied from 2 to 10 atoms, showed that the rate of reaction could be enhanced greatly by the appropriate choice of group.[24] Complex formation with the maleimide varied greatly (factor of over 100) according to the length of the alkyl group, *N*-*n*-decylmaleimide being most strongly bound prior to reaction with the active site thiol group.

Conclusions. Both reactivity and specificity can be regulated in maleimide derivatives. The development of maleimides and related compounds as biological control agents and receptor site labels should lead to interesting and useful results.

§ N. S. Kosower, personal communication.

T. Miyadera and B. Dangerfield, unpublished results.

Experimental Section

Nmr measurements were made with an A-60 spectrometer. Ir spectra were taken with a Perkin-Elmer 457 spectrophotometer and uv spectra with a Cary Model 14.

Maleimides. *N*-Ethyl-2-methyl (1). $EtNH_2$ (2.5 g, 55 mmoles) in 10 ml of C_6H_6 was added to 2-methylmaleic anhydride (citraconic anhydride, 5.6 g, 50 mmoles) in C_6H_6 (20 m), xylene (100 ml) was then added, and the solvents were evapd slowly at atm press, and finally under vacuum. The residue distd as a colorless liquid, bp 93° (13 mm), which was redistd to give 4.72 g (68%) of colorless liquid.

***N*-Ethyl-2,3-dimethyl (2).** ***N*-Ethyl-3,4,5,6-tetrahydrophthalimide (3)** was reported previously.[1] ***N*-(2-Hydroxyethyl)-2,3-dimethylmaleimide (2H)** was prepd as for 1.[1]

Bicyclo[2.2.1]hept-2-ene-2,3-dicarboximide (4). The anhydride (from the diacid and Ac_2O[25]) was converted into the monoethylamide by the procedure shown for 1 and was obtd as colorless cryst from EtOAc, mp 184–185°. The imide was obtd by heating the amide (941 mg) and P_2O_5 (3.0 g) in DMF (10 ml) at 110° for 2 hr with stirring. Et_2O and H_2O were added, the Et_2O later washed, dried, and evapd, and the residue distd to give a colorless oil (150 mg) which solidified on standing. Recrystallization from petr ether gave colorless cryst, mp 38–39°. **Showdomycin (8)** was purchased from Calbiochem. 2-Methylmaleimide (9) was synthesized by the method of Protopopescu, *et al.*[26]

Barbiturate Derivatives. The anthracene adduct of 2-methylmaleic anhydride[27] was reacted with 2-aminoethanol in acetone, xylene was added, and the mixture treated as above for 1. The succinimide, mp 144–145°, was recrystd from C_6H_6 and obtd in 64% yield. The corresponding amide from 2-(2-hydroxyethoxy)ethylamine, mp 137–138° (from C_6H_6), was obtd in a similar fashion. The tosylate derivatives were generated with *p*-TsCl and pyridine,[1] and were oils for which the structures were confirmed by nmr. The barbiturate–anthracene adducts were prepd by the procedure already described,[1] purified by chromotography, and pyrolyzed under reduced pressure (40–50 mm) at 250°, using a liquid N_2 cooled cold finger to trap the barbiturate. The sublimate was purified by chromatog. Compd **5**, mp 111–112°, and **6**, mp 95–96°, were prepd in a similar procedure.

Kinetic Studies. Solns of the maleimides (4 was first dissolved in 0.1 ml of MeCN and mixed with 10 ml of buffer) in 0.5 *M* phosphate buffer were deoxygenated by alternate exposure to vacuum and N_2 (5–6 cycles) or, for the most reactive compds, by a stream of argon passed through the soln contd in a 10-cm cell. The soln was contd in an app with an optical cell at the bottom and a side arm contg the appropriate amt of glutathione. At $t = 0$, the buffer soln was mixed with the GSH to begin the reaction, shaken briefly to ensure soln and placed in the light path of the sample compartment of a Cary Model 14 spectrophotometer. The reaction was followed at a particular wavelength (see Table I) until the rate of change was low enough to permit taking complete spectra. The optical densities at a single wavelength were used to compute a rate constant with a second-order rate equation and an IBM Model 1800 computer. The results are recorded in Table I. A different equation was used for the reaction of GSH with **2**, since the reaction does not go to completion but reaches an equil contg about 90% adduct. (See Product Studies below.)

Product Studies. Reaction of *N*-Ethyl-2-methylmaleimide (1) with GSH. GSH (110 mg, 3.58 mmoles) was added to a suspension of 1 (50.5 mg, 0.360 mmole) in 0.5 *M* phosphate buffer, pH 7.68 (0.5 ml), the pH adjusted with solid Na_2CO_3 to about 7, and the nmr spectrum taken. The soln became homogeneous after the addn of GSH. As expected on the basis of the rate constant, the reaction was complete before the nmr spectrum was taken. The adduct spectrum contd an intense singlet at 31.5 cps lower field than Me protons of the *N*-Et in place of the doublet assigned to the Me attached to the double bond of the maleimide. The nmr spectrum of the maleimide in CCl_4 has the following peaks: τ 8.88 (CH_2CH_3, t, J = 7.1 cps), 6.58 (CH_2CH_3, q, J = 7.1 cps), 7.99 (CH_3, d, J = 1.8 cps), 3.83 (=CH, q, J = 7.1 cps). The *N*-ethyl-2,3-dimethylmaleimide (**2**) did not have sufficient solubility for a product study and was replaced by the *N*-(2-hydroxyethyl) derivative (**2H**). The imide **2H** (50.3 mg, 0.300 mmole) was dissolved in 0.5 *M* phosphate buffer, pH 7.68 (0.5 ml), GSH (101 mg, 0.329 mmole) added, and the pH adjusted to 7 with Na_2CO_3. Nmr spectra were taken at intervals, with equil reached only 5 hr after mixing. The integrated Me signal intensities, which differed in the imide and the adduct, showed a final ratio of 9:1 for adduct–imide **2H**; nmr **2H**: τ 8.08 ($2CH_3$, s), 6.57 (OH,s), 6.43 (CH_2CH_2, broad triplet). Adduct. Nmr showed singlet at 17.5 cps higher field than CH_3 of **2H**, doublet at 39 cps (J = 7.1 cps) higher than CH_3 of **2H**.

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References

(1) T. Miyadera, E. M. Kosower, and N. S. Kosower, *J. Med. Chem.*, 14, 873 (1971) (paper 1), and references cited therein.
(2) W. A. Pryor, "Mechanisms of Sulfur Reactions," McGraw-Hill Book Co., New York, N. Y., 1962, pp 6, 60.
(3) F. Schneider and H. Wenck, *Hoppe-Seyler's Z. Physiol. Chem.*, 350, 1521 (1969); G. Gorin, P. A. Martic, and G. Doughty, *Arch. Biochem. Biophys.*, 115, 593 (1966); A. Karlin and M. Winnik, *Proc. Nat. Acad. Sci. U. S.*, 60, 668 (1968).
(4) E. M. Kosower and N. S. Kosower, *Nature (London)*, 224, 117 (1969).
(5) E. M. Kosower, "Introduction to Physical Organic Chemistry," Wiley, New York, N. Y., 1968.
(6) J. Hine, "Physical Organic Chemistry," 2nd ed, McGraw-Hill, New York, N. Y., 1962.
(7) R. B. Turner, W. R. Meador, and R. E. Winkler, *J. Amer. Chem. Soc.*, 79, 4116 (1957).
(8) R. J. Suhadolnik, "Nucleoside Antibiotics," Wiley-Interscience, New York, N. Y., 1970, p 393.
(9) E. M. Kosower and T. Miyadera, *J. Med. Chem.*, 15, 307 (1972).
(10) M. Beljanski, P. Bourgarel, and M. Beljanski, *Proc. Nat. Acad. Sci. U. S.*, 68, 491 (1971).
(11) S. Roy-Burman, Y. H. Huang, and D. W. Visser, *Biochem. Biophys. Res. Commun.*, 42, 445 (1971).
(12) E. M. Kosower, W. Correa, B. J. Kinon, and N. S. Kosower, *Biochim. Biophys. Acta*, in press (1972).
(13) S. Roy-Burman, P. Roy-Burman, and D. W. Visser, *Cancer Res.*, 28, 1605 (1968).
(14) L. Kalvoda, J. Farkaš, and F. Šorm, *Tetrahedron Lett.*, 2297 (1970).
(15) K. Karyone, Japanese Patent 6900536; *Chem. Abstr.*, 70, 77624 (1969).
(16) F. Reicheneder, K. Dury, E. H. Pommer, and H. Stummeyer, German Patent 1147006; *Chem. Abstr.*, 59, 11289 (1963).
(17) B. S. Janes and J. Kuiper, *Aust. J. Exp. Agr. Anim. Husb.*, 3, 209 (1963).
(18) J. A. W. Turner, J. N. Turner, and G. H. F. Walker, British Patent 880,555; *Chem. Abstr.*, 56, P13299 (1962).
(19) F. Reicheneder, K. Dury, H. Adolphi, and H. Stummeyer, German Patent 1146694; *Chem. Abstr.*, 59, P3277*f* (1963).
(20) R. L. Gates and K. P. Dorschner, U. S. Patent 2865730; *Chem. Abstr.*, 53, 6517 (1959).
(21) J. F. Cavins and M. Friedman, *J. Biol. Chem.*, 243, 3357 (1968).
(22) M. Friedman, J. F. Cavins, and J. S. Wall, *J. Amer. Chem. Soc.*, 87, 3672 (1965).
(23) E. Boyland and L. F. Chasseaud, *Biochem. J.*, 104, 95 (1967).
(24) B. M. Anderson and E. C. Vasini, *Biochemistry*, 9, 3348 (1970).
(25) O. Diels, K. Alder, and H. Nienberg, *Justus Liebigs Ann. Chem.*, 490, 236 (1931).
(26) L. Protopopescu, *et al.*, *Soc. Chim. Romania, Sect. Rom. Stiinte, Bul. Chim. Pura Apl.*, 2, 71 (1940); *Chem. Abstr.*, 38, 3958 (1944).
(27) W. E. Bachmann and L. B. Scott, *J. Amer. Chem. Soc.*, 70, 1458 (1948).