The Reactions of Amines and Amino Acids with Maleimides. Structure of the Reaction Products Deduced from Infrared and Nuclear Magnetic Resonance Spectroscopy*

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ABSTRACT: It has been recognized that *N*-ethylmaleimide can react with amino groups in proteins under the same conditions which are often used to alkylate sulfhydryl groups. However, the nature of the reaction has not been unequivocally established by isolation of reaction products. An investigation of the reactions of maleimides with simple amines and amino acid derivatives was prompted by this consideration, and independently by the discovery that maleimides can react with a transient intermediate in the decomposition of certain amino acids catalyzed by pyridoxal phosphate enzymes. Products were isolated from the reactions of maleimides with pyrrolidine and piperidine, of *N*-phenylmaleimide with benzylamine and glycylamide, and of *N*-ethylmaleimide with piperidine, glycylamide, and L-proline.

The two diastereoisomers formed from L-proline in the latter reaction were separated by electrophoresis and isolated as crystalline picrates. Infrared and nuclear magnetic resonance spectroscopy of these compounds indicated that in every case the reaction involved addition of the amino group to the maleimide double bond. In neutral aqueous solution proline reacted much faster than primary amino acids with similar amino group ionization constants. Also, in contrast to amino acids containing the primary amino group, the reaction of proline with maleimides was reversible under various conditions, proline being regenerated by heating in the solid state, and in solution in strong acid, and probably also at pH 5. In addition, a search was made, with negative results, for charge-transfer complex formation between maleimides and compounds electronically related to pyridoxal phosphate.

This study of the reactions between maleimides and amines or amino acids was prompted in part by the discovery that maleimides, and various *N*-substituted derivatives, can react with and trap a transient intermediate in pyridoxal phosphate potentiated enzymatic elimination reactions from substituted C-4 amino acids (Flavin, 1965a,b). Since the reaction intermediate was originally postulated to be vinylglycine (Flavin and Slaughter, 1964), and this compound had not been prepared, it became important to explore the potentiality for reaction between compounds related to it, and maleimides.

A more widely shared interest lies in the realization, arising from the widespread use in recent years of *N*-ethylmaleimide as a protein sulfhydryl blocking agent, that *N*-ethylmaleimide can also react to a significant extent with protein amino groups. In spite of the interest in this subject, little information has been available on the rates of reaction of maleimides with amines and amino acids, or on the products of the reactions. At the time this work was undertaken, opinion was divided as to whether the reactions involved amine addition to the double bond (Tawney *et al.*, 1961), or amine attack on the imide carbonyl (Smyth *et al.*, 1960). Since then the proponents of the latter view have obtained further evidence which favors addition to the double bond (Smyth *et al.*, 1964; Guidotti and Konigsberg, 1964). The current consensus rests, however, on indirect evidence. In no case has the product of a reaction of an amino acid derivative been isolated and, to our knowledge, the only amine from which a reaction product has been isolated is piperidine (Tawney *et al.*, 1961; Mustafa *et al.*, 1961). The structure of the latter reaction product was not determined, but was inferred from a limited analogy to extensively studied additions of amino compounds to α,β-unsaturated ketones (Cromwell, 1946).

This report describes the isolation and structure determination of the products arising from the reaction of certain primary and secondary amines and amino acids with N-substituted maleimides, as well as some preliminary rate studies of these reactions.

Experimental Section

Chemical Preparations. PYRROLIDINE AND MALEIMIDE. Pyrrolidine (4.9 mmoles) was added to 20 ml of benzene saturated with maleimide (4.7 mmoles). After 5 min at 25° the reaction was complete, as judged by

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a decrease of the maleimide absorbancy at λ 276 mμ, to 3% of its initial value. Helium was bubbled through the solution to reduce the volume to 1 ml. Addition of petroleum ether (bp 30–60°) precipitated an oil which solidified on chilling: 675 mg (87%), mp 119–124°. The product I was recrystallized from ethyl acetate–CCl_4, mp 123–125°.

PIPERIDINE AND MALEIMIDE. This reaction product was isolated in two forms with different melting points. By the procedure described for pyrrolidine, large flake crystals of mp 117–118° were isolated in 87% yield (IIa). This form was unstable, and sometimes showed a rise in the melting point after storage in the solid state. When heated in benzene it was converted in 50% yield to a less soluble product IIc, mp 197–198.5°, which was found on analysis to contain an extra molecule of oxygen, but for which no structure was established. When the mp 118° form was dissolved in ethyl acetate, addition of CCl_4 precipitated a stable form IIb, mp 147°, in large thin rectangular plates. This form has been reported previously (Tawney *et al.*, 1961; Mustafa *et al.*, 1961), mp 142.5°. Once this form had been obtained, the low-melting form could not again be isolated in the same laboratory until after a lapse of time.

Piperidine picrate was obtained by adding a small excess of amine to 2 mmoles of picric acid dissolved in 8 ml of warm ethanol. The precipitate of small needles which separated on chilling was washed with ethanol and ether, mp 153.5–154.5°. The picrate of the piperidine–maleimide product (mp 147°) was obtained in 88% yield in the same way. Recrystallization from ethyl acetate–ethanol yielded thick hexagonal plates, mp 172–174° (IIb′). The product III of reaction of piperidine with *N*-ethylmaleimide was also isolated, mp 39–40°.

BENZYLAMINE AND *N*-PHENYLMALEIMIDE. Benzylamine and *N*-phenylmaleimide (1 mmole each) were dissolved in 2 ml of benzene, which was warmed occasionally over a 2-hr period. After discarding a precipitate which formed on chilling, the solution was concentrated to 0.5 ml. Addition of petroleum ether precipitated 250 mg of product, mp 75–85°. Impurities were separated by solution in hot CCl_4 followed by rapid chilling and filtration of the precipitate: 163 mg (58%), mp 102–103°. Slow crystallization of the latter from CCl_4 yielded transparent prisms with unchanged melting point (IV).

GLYCYLAMIDE AND *N*-ETHYLMALEIMIDE. Glycylamide was prepared from its hydrochloride by the procedure of Bergell and Wulfing (1910). After recrystallization from $CHCl_3$ the yield was 46%, mp 65–67°. To 2 mmoles of glycylamide dissolved in 16 ml of hot $CHCl_3$ was added 2 mmoles of *N*-ethylmaleimide in a small volume of $CHCl_3$, followed by 6 ml of benzene. After refluxing for 2 days, the mixture was chilled and the reaction product was filtered out. One recrystallization from $CHCl_3$–benzene yielded 113 mg (28%) of flaky crystals, mp 139–140.5° (V).

GLYCYLAMIDE AND *N*-PHENYLMALEIMIDE. After 20 hr of reflux under the conditions used for *N*-ethylmaleimide, and after discarding a precipitate which formed at reflux temperature, 178 mg (36%) of crude product, mp 128–132°, was obtained. Reprecipitation from hot $CHCl_3$ containing a few per cent of methanol yielded 90 mg of an amorphous colorless product VI, mp 137–138°.

L-PROLINE AND *N*-ETHYLMALEIMIDE. L-[U-^{14}C]Proline (1 mmole) (2×10^8 cpm) was dissolved in 0.1 ml of 10 N KOH. Into this, at 25°, 0.4 ml of acetonitrile containing 1 mmole of *N*-ethylmaleimide was forcibly injected from a syringe. The mixture was quickly shaken until it became homogeneous (30 sec), and was then left at 25° for 30 min. After dilution with 1 ml of water and acidification to pH 3 with HCl, the mixture was applied to a 1×130 cm column of Dowex $50H^+$ in the hope of separating the expected diastereoisomers. The column was eluted with a linear gradient; the mixing vessel contained 800 ml of 2.5 N HCl and the reservior the same volume of 5.5 N HCl. The added radioactivity (98%) was recovered in a single sharp peak in the fractions from 3.3 to 3.9 N HCl. The residue obtained after evaporation of these combined fractions was a hygroscopic glass. The picrate salt of the latter was prepared by acidifying an ethanolic solution to pH 2 with ethanolic picric acid. The filtered solution was evaporated to an oil which was dissolved in ethyl acetate. Addition of CCl_4 precipitated a yellow, amorphous but nonhygroscopic solid VII, with no sharp melting point.

The two diastereoisomers present in the above crude reaction product could be separated by high-voltage paper electrophoresis (Flavin and Slaughter, 1966) in 4% formic acid, pH 1.7. In 16 hr at 3500 v component 1, the stronger acid, migrated 24 cm toward the cathode, and component 2, which represented two-thirds of the material (by radioassay), migrated 52 cm. An attempt was made to isolate these components. Ethanol eluates from a large number of electrophoretic papers were concentrated and treated with a calculated amount of picric acid, and crystals were obtained from ethyl acetate after several days in the cold. Component 1 was obtained as irregular yellow crystals, mp 122–124°, and component 2 as large rectangular prisms, mp 130–135° (the values are for transition from solid to viscous liquid). By paper electrophoresis each component was radiographically homogeneous and had undergone no conversion to the other. However, poor results were obtained from elemental analyses.

Analytical Procedures and Materials. The rates of reaction of maleimides with a number of amines and amino acids were determined at 25° in water buffered with 0.1 M Tris-HCl at pH 7.3 or 8.5. Only initial rates were measured, and these are recorded in Table I as the time required for disappearance of 10% of the added maleimide in the presence of 0.01 M amino compound, in 1-ml volume. Rates were corrected for the hydrolytic decomposition of maleimide in the absence of amine. At pH 8.5 the $t_{1/10}$ for hydrolysis was 15 min for *N*-ethylmaleimide and 12 min for maleimide. Slow reactions with amines could there-

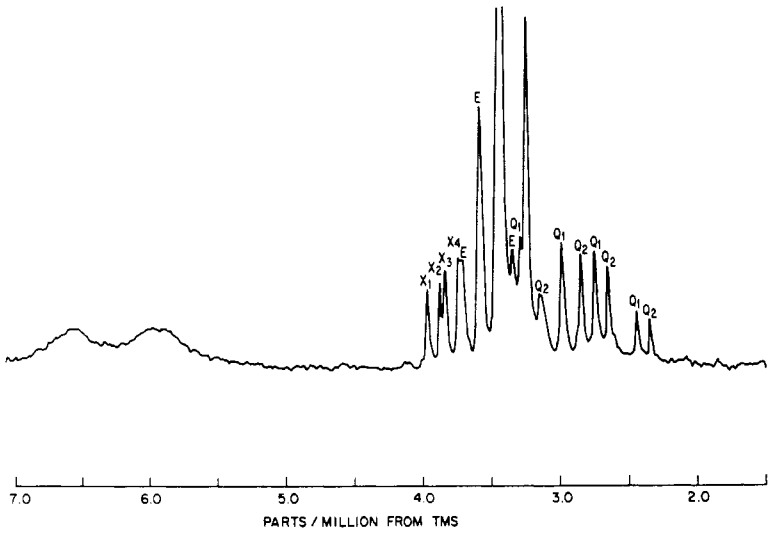


FIGURE 1: Nuclear magnetic resonance spectrum of *N*-ethylmaleimide–glycylamide adduct in liquid SO_2 at 22°. Q_1, Q_2 and X labels are to the ABX system, while the E labels are to the CH_2 protons of the *N*-ethyl substituent. The band at 3.42 ppm is due to the CH_2 of the glycylamide portion, while the band at 3.24 ppm is due to the NH group. The fourth E component is obscured by the band at 3.42 ppm.

TABLE I: Analytical Results on Adducts.

Compd	Amine	Male-imide	Formula	Mp (°C)	Pic-rate	Calcd C	Calcd H	Calcd N	Found C	Found H	Found N
I	Pyrrolidine	H	$C_8H_{12}N_2O_2$	123–125.5	...	57.12	7.19	16.66	57.04	6.89	16.82
IIa	Piperidine	H	$C_9H_{14}N_2O_2$	118	...	59.32	7.74	15.38	59.40	7.38	15.95
b	Piperidine	H	$C_9H_{14}N_2O_2$	147	...	59.32	7.74	15.38	59.82	7.81	15.06
c	Piperidine	H	*a*	197	...				50.27	6.69	13.18
b′	Piperidine	H	$C_{15}H_{17}N_5O_9$	172–174	√	43.80	4.17	17.03	43.73	4.15	17.14
IV	Benzylamine	Phenyl	$C_{17}H_{16}N_2O_2$	102–103	...	72.84	5.75	9.99	72.93	5.79	9.83
V	Glycylamide	Ethyl	$C_8H_{13}N_3O_3$	139–140.5	...	48.25	6.87	21.10	48.14	6.26	19.98
VI	Glycylamide	Phenyl	$C_{12}H_{13}N_3O_3$	137–138	...	58.28	5.30	16.99	58.02	5.51	16.96
VII	Proline	Ethyl	$C_{17}H_{19}N_5O_{11}$	...	√	43.50	4.08	14.92	43.50	4.29	12.93
	Piperidine	...	$C_{11}H_{12}N_2O_7$		√	42.04	4.49	17.83	41.96	4.30	17.96

[a] This high melting material obtained by refluxing in benzene seems to fit best the formula $C_9H_{14}N_2O_4$.

fore not be measured accurately. All reactions were followed by measuring the decrease in the long-wavelength absorption bands of the maleimides. These bands disappear after reactions at either the double bond or the carbonyl functions of maleimides (Flavin and Slaughter, 1964).

Separation of proline from its reaction products with maleimides was effected with the following paper chromatographic solvents: (1) *t*-butyl alcohol–90% formic acid–water, 70:15:15, descending; (2) ethyl alcohol–water–concentrated NH_4OH, 7:1:2, ascending; (3) cyclohexylamine–methyl ethyl ketone–*n*-butyl alcohol–water, 15:75:75:37.5, ascending. *N*-Phenyl-, *N*-vinyl-, *N*-hydroxymethyl-, and *N*-chloromethylmaleimide, butadiene sulfone, and *N*,*N*′-dimethylmaleic hydrazide were obtained from Dr. P. O. Tawney, and 3,4-dehydro-DL-proline was a gift from Dr. B. Witkop.

Results

The elemental analyses of the various adducts of maleimides with the amino compounds were consistent with the formation of a compound containing one molecule of each of the starting materials, and no apparent loss of small molecules (Table I). Infrared spectra were run on these adducts, either in KBr pellet or Nujol mulls. The data relevant to the various amide carbonyl bands for five-membered cyclic imides (Table II) are at *ca.* 1770 and 1700 cm^{-1} (Nakanishi, 1962; Uno and Machida, 1962; Matsuo, 1964).

TABLE II: Infrared Vibrations, Carbonyl Bands.

Compd	Amine	Maleimide N Substitution	Matrix	Amide I (cm^{-1})	Amide II (cm^{-1})	Cyclic Imide (cm^{-1})
I	Pyrrolidine	H	KBr	1715	...	1771
IIa	Piperidine	H	Nujol	1712	...	1770
IV	Benzylamine	Phenyl	KBr	1709	...	1779
V	Glycylamide	CH_3CH_2	KBr	1706, 1672	1634	1779
VI	Glycylamide	Phenyl	KBr	1718, 1681	1603	1786
...		CH_3CH_2[a]	KBr	1715		1776
...	Glycylamide HCl	...	KBr	1695	1595	...

[a] Also broad band at 1634 cm^{-1} due to double bond.

In addition to these bands, *N*-ethylmaleimide also shows a broad, weak band at 1634 cm^{-1} due to the double-bonded carbon atoms. Since the bond is conjugated to the carbonyl systems, it should appear in the 1650- to 1600-cm^{-1} region (Nakanishi, 1962). All compounds investigated contained these features of the cyclic imide, and showed no absorption attributable to an ethylenic system. Compounds V and VI contained the primary amide grouping of glycylamide, having retained that grouping as evidenced by the presence of an amide II band in the infrared spectrum and two NH bands in the nmr[1] spectrum (due to restricted rotation about the CN bond) (Pople *et al.*, 1959). The ultraviolet spectrum of the glycylamide–*N*-ethylmaleimide reaction product in water showed a shoulder in the end absorption at λ 245 mμ, molar absorbancy of 250, lost after raising the pH to 11 and returning it to neutrality. This result is consistent with the presence of an intact succinimide ring (Smyth *et al.*, 1964).

Nuclear magnetic resonance studies of these adducts (Table III) in various solvents ($CDCl_3$, SO_2, D_2O, etc.) showed invariably the loss of the bands due to the vinyl protons of the maleimide (*ca.* 6.7 ppm; Table IV) and the appearance of new groups of bands which could be ascribed to the protons of an ABX system, or an A_2X system. In view of the structures postulated for the adducts, the A_2X sets are undoubtedly examples of "deceptively simple spectra" (Diehl, 1963). Other specific groups in the original molecules remained unchanged in the adducts (*N*-ethyl groups in the maleimides, etc.). All or part of any hydrogen attached to the amino nitrogen was lost, indicating no reaction at the nitrogen of the maleimide but, correspondingly, reaction at the amino nitrogen. Representative complete nmr spectra are shown in Figures 1 and 2. All of the spectroscopic properties of the products of reaction between maleimides and these various amines and amino acids are consistent with their having the structures of substituted succinimides, resulting from addition of the amino group to the double bond of the maleimide.

The product of the reaction between piperidine and maleimide was isolated in an unstable crystalline form IIa, mp 118°, which on storage or recrystallization reverted to the stable form IIb, mp 147°, which had been reported previously. The elemental analysis for IIa was essentially the same as for IIb (Table I), and its nmr spectrum was nearly identical with that of IIb (Table III), but showed in addition two small peaks (each less than 1 proton, based on the formula in Table I) at 6.70 ppm. These peaks might conceivably be due to complexed or contaminating maleimide and/or benzene. Refluxing of IIa in benzene yielded a third product IIc, mp 197°, which by analysis appeared to contain an extra molecule of oxygen (Table I), but no structural determinations were carried out on this compound.

The picrate of the product of the reaction of L-proline with *N*-ethylmaleimide (VII) was too insoluble in most solvents for adequate nmr. However, the rather poorly defined spectrum obtained in deuteriodimethyl sulfoxide was in accord with the spectra of the other adducts, and indicated addition of the amino acid nitrogen to the maleimide double bond. Overlapping of the signals of the succinimide protons with those of the protons on the carbon atoms adjacent to the nitrogen atom of the proline prevented complete resolution.

Table V shows the results of a preliminary survey of the rates of reaction of maleimides with various amino compounds, in water at 25°, at pH 7.3 or 8.5. The listed pK_a values are from the literature except for those of glycylamide, which was determined by titration, and of dehydroproline, for which we are indebted

[1] Abbreviations used: nmr, nuclear magnetic resonance; TMS, tetramethylsilane; P, phosphate.

TABLE III: Nuclear Magnetic Resonance Data.

Compound	I	IIb	IV	V	V	V	V	V	V	V	VI	VI
Amine	Pyrrolidine	Piperidine	Benzylamine	Glycylamide	Glycylamide	Glycylamide	Glycylamide	Glycylamide	Glycylamide	Glycylamide	Glycylamide	Glycylamide
Maleimide, N substituents	H	H	Phenyl	Ethyl	Ethyl	Ethyl	Ethyl	Ethyl	Ethyl	Ethyl	Phenyl	Phenyl
Solvent	$CDCl_3$	$CDCl_3$	$CDCl_3$	D_2O	SO_2	SO_2	SO_2	SO_2	SO_2	SO_2	D_2O	SO_2
Temperature, °C	Room	Room	Room	22	Room	22	0	−20	−40	−60	+60	22
Pyrrolidine, ν_1[a]	2.96	...	...	...	...	...	...	...	...	...	...	...
Pyrrolidine, ν_2	1.83	...	...	...	...	...	...	...	...	...	...	...
Piperidine, ν_1	...	1.53	...	...	...	...	...	...	...	...	...	...
Piperidine, ν_2	...	2.78	...	...	...	...	...	...	...	...	...	...
Ethyl, νCH_3	...	...	...	1.13	1.16	1.13	1.13	1.13	1.14	1.15	...	...
Ethyl, νCH_2	...	...	...	3.55	3.50	3.52	3.56	...	...	...	...	...
Ethyl, J_{AB}	...	...	...	7.3	7.3	7.3	7.4	7.5	7.5	7.3	...	...
Three-spin, νA	...	...	2.79	2.81	2.74	2.74	2.84	...	...	...	3.03	2.95
Three-spin, νB	...	...	2.90	2.88	2.85	2.86	2.97	...	...	...	3.15	3.07
Three-spin, νX	3.85	3.90	3.91	3.97	3.85	3.85	3.88	...	...	...	4.19	4.09
Three-spin, J_{AB}	14.0	14.0	17.8	18.5	18.3	18.5	18.8	...	...	...	17.8	18.0
J_{AX}	...	...	8.6	8.0	8.6	8.9	8.6	...	...	...	8.5	8.9
J_{BX}	...	...	5.0	5.5	4.9	4.6	5.4	...	...	...	5.0	5.1
CH_2, ν	...	...	3.92	3.52	3.42	3.42	3.52	3.63	3.67	3.59	3.59	3.51
NH, ν	...	...	2.20	...	3.02	3.24	4.28	4.88	5.21	5.45	...	...
Phenyl group, ν	...	...	7.33	...	...	...	...	...	...	...	7.50	7.63
Amide 1, ν	...	...	...	...	...	5.96	6.17	6.27	6.37	6.45	...	...
Amide 2, ν	...	...	...	...	...	6.58	6.71	6.72	6.73	6.48	...	...

[a] Frequencies (ν) in parts per million from TMS (δ values); coupling constants (J) in cycles per second.

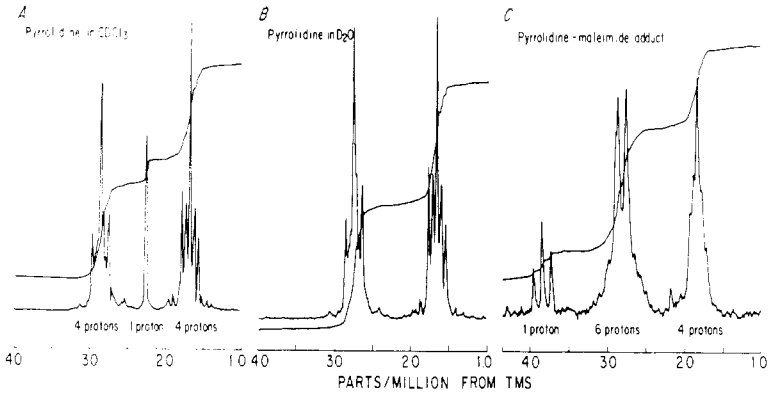


FIGURE 2: Pyrrolidine in $CDCl_3$ and D_2O together with its maleimide adduct in $CDCl_3$.

TABLE IV: Nuclear Magnetic Resonance of Maleimides and Related Compounds.

Compound	Solvent	Vinyl Proton (ppm) from TMS	Other Protons (ppm) from TMS
Maleimide	$CDCl_3$	6.72, 6.70 [a]	
N-Ethylmaleimide	SO_2	6.68	CH_3 1.12; CH_2 3.49 (J = 7.0)
N-Phenylmaleimide	D_2O	7.01	Phenyl group *ca.* 7.42
N-Vinylmaleimide	D_2O	6.88	N-Vinyl protons *ca.* 5.02, 5.68, and 6.63
N-Hydroxymethylmaleimide	D_2O	6.92	Methylene 5.00
N-Chloromethylmaleimide	D_2O	6.92	Methylene 5.00
Butadiene sulfone	D_2O	6.12	Methylene 3.88
N,N'-Dimethylmaleic hydrazide	D_2O	7.07	Methyl 3.68

[a] Doublet due to coupling with amide proton; J = 1.3 cycles/sec.

to Dr. B. Witkop; the values for homoserine and allylglycine, which may be considered typical neutral amino acids, were not available. Homoserine lactone (Flavin and Slaughter, 1964) was shown by hydroxylamine assay not to undergo ring opening during the reaction period. L-Proline methyl ester reacted more rapidly than proline; these results are not shown because the ester was rapidly hydrolyzed during the reaction at pH 8.5.[2]

Because the rapidity of the reaction of proline and its acyl derivatives with maleimides is of interest from the standpoint of the use of *N*-ethylmaleimide in investigating protein structure and function, additional studies of this reaction were carried out. The product of this reaction when treated with 6 N HCl at 110° for 48 hr in sealed vials regenerated proline (showed by paper chromatography). Strong acids thus reverse the reaction of proline with maleimides.

The product of the reaction of proline with *N*-ethylmaleimide was found to give a good yield of the yellow color characteristic for proline, when treated with ninhydrin by the Moore–Stein procedure. This was puzzling since tertiary amines do not react with ninhydrin (Johnson and McCaldin, 1958). With the Chinard (1952) ninhydrin procedure (60 min at 100° in 1 N H_3PO_4 in acetic acid) proline gives an intense red color, whereas the proline–maleimide reaction product gave no color. It was found that when the reaction product was first exposed to the Moore–Stein reaction conditions (20 min at 100° at pH 5) in the absence of ninhydrin, and then assayed by the Chinard procedure, it gave the red color calculated for complete decomposition to proline during the prior reaction at pH 5. These results suggest that the starting material is also regenerated from the reaction products of proline and maleimide, by heating the latter at pH 5.

It had been reported that addition of a secondary amine to a maleimide could be reversed by heating

[2] We were not able to obtain crystals of L-proline methyl ester hydrochloride by the procedure of Erlanger *et al.* (1954). A crystalline derivative, mp 121–121.5°, was isolated, however, which appeared to be (proline methyl ester)$_2$·HCl·$HClO_4$. *Anal.* Calcd: C, 36.46; H, 6.12; N, 7.08; total Cl, 17.94; ionic Cl, 8.97. Found: C, 36.61; H, 6.08; N, 7.37; total Cl, 18.19; ionic Cl, 8.82.

TABLE V: Initial Rates of Reaction of Maleimides with Amines and Amino Acids.[a]

Maleimide Derivative	Concn (M)	Amino Compd (all 0.01 M)	Amino pK_a	Reaction pH	Initial Reaction Rate (time for 10% disappearance of maleimide), min	k_2 (l. sec^{-1} $mole^{-1}$)
N-Ethylmaleimide	0.001	3,4-Dehydro-DL-proline	9.6	8.5	1.3	0.14
N-Ethylmaleimide	0.001	Piperidine	11.2	8.5	1.7	0.10
N-Ethylmaleimide	0.001	L-Proline	10.6	8.5	3	0.059
N-Ethylmaleimide	0.001	Benzylamine	9.4	8.5	7	0.025
N-Ethylmaleimide	0.001	Glycylglycine	7.7	8.5	8	0.022
N-Ethylmaleimide	0.001	Glycylamide	7.8	8.5	9	0.020
N-Ethylmaleimide	0.001	Diethylamine	11.0	8.5	27	0.0065
N-Ethylmaleimide	0.001	DL-Homoserine		8.5	60[b]	...
N-Ethylmaleimide	0.001	DL-Homoserine lactone		8.5	100[b]	...
N-Ethylmaleimide	0.001	DL-Allylglycine		8.5	*b*	...
N-Ethylmaleimide	0.001	Aniline	4.6	8.5	*b*	...
N-Ethylmaleimide	0.001	L-Proline	10.6	8.5	3	0.059
N-Ethylmaleimide	0.001	L-Proline	10.6	7.3	28	0.0063
Maleimide	0.0015	Piperidine	11.2	8.5	8	0.022
Maleimide	0.0015	L-Proline	10.6	8.5	9	0.020
Maleimide	0.0027	L-Proline	10.6	8.5	8	0.019

[a] Corrected for maleimide hydrolysis. Conditions for reactions and measurements of rates are given in the text. [b] Not reliably measureable.

the adduct above its melting point; thus *N*-phenylmaleimide was recovered after heating its piperidine adduct for 1 hr at 150° (Mustafa *et al.*, 1961). The crystalline picrate of component 1 (see Experimental Section) of the proline *N*-ethylmaleimide reaction product, mp 122–124°, was placed without solvent in a 140° bath for 20 min. After paper electrophoresis 91% of the original radioactivity was recovered. Of this, 60% was proline, 26% was unchanged component 1, and 14% was component 2.

Finally, a series of experiments was carried out, unrelated to the other work reported here, to seek evidence for complex formation between maleimide and compounds related to pyridoxal P. These were prompted by the isolation of a charge-transfer complex between maleimide and resorcinol (Tawney *et al.*, 1961), and by the discovery, mentioned in the introduction, that maleimides could react with a transient intermediate in certain pyridoxal P dependent enzymatic elimination reactions. No spectral evidence was obtained for any complex formation between maleimides and pyridoxal or salicylaldehyde. However, the latter may not be the best model systems for all possible enzyme-bound forms of the coenzyme.

Discussion

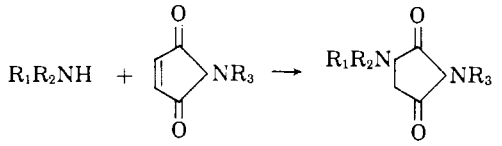
Smyth *et al.* (1960) first called attention to the fact that *N*-ethylmaleimide can react appreciably with certain free amino acids, and more generally with amino acid amide derivatives, in the physiological pH and temperature range which is used for reaction of maleimide with protein sulfhydryl groups. Cystine was among the reactive free amino acids, and it was found that cystathionine and lanthionine also react to yield chromatographically separable ninhydrin-reactive products under these mild conditions (Flavin and Slaughter, 1964). The reactivity of these amino acids was attributed to the relatively weak basicity of one of their two amino groups ($pK_a = 8$), allowing a sufficient fraction of that amino group to be unprotonated at neutral pH and available for attack on the maleimide.

A few preliminary rate measurements, in buffered water (Table V), were undertaken to aid in selecting the amino compounds from which to isolate reaction products. Under these conditions the importance of the fraction of amino group which is unprotonated is suggested by the effects of pH, and the comparative rates with proline and dehydroproline, and glycylglycine and allylglycine. This factor obscures the importance of base strength, as indicated by the fact that in organic solvents the piperidine reaction was complete in 5 min at 25°, whereas only a modest yield of glycyclamide reaction product was obtained after 2 days of reflux.

The results of Table V also suggest some other structural factors affecting reaction rates.

A plausible mechanism for the reactions would consist of a 1,4 addition of the amine to the dipolar form of the maleimide, giving rise to the enol form of the product, which ketonizes to the final product. The electron-releasing effect of the ethyl group, compared to hydrogen, would reinforce the electron density at one of the carbonyl groups and account for the faster rates with *N*-ethylmaleimide.[3]

The characteristics of the infrared and nuclear magnetic resonance spectra of all the reaction products were consistent with addition of the amino group to the double bond of the maleimide



where R_1 and R_2 may be carbon atoms in a ring (piperidine, pyrrolidine) or an additional hydrogen atom (benzylamine, glycylamide) and R_3 may be hydrogen, ethyl, or phenyl. If the adding compound contained a primary amino group, the resulting three-proton system of the succinimide gave rise to an ABX-type spectrum, with $|J_{AB}|$ approximately 18 cycles/sec, in agreement with other geminal protons in this type orientation (Barfield and Grant, 1963; Banwell and Sheppard, 1962; Bothner-By, 1965); the sign of J_{AB} is probably negative. J_{AX} was found to be about 8.6 cycles/sec and J_{BX} about 5.0 cycles/sec (Pople *et al.*, 1959; Bible, 1965, for the methods of calculation), and are therefore positive. J_{AX} is then the *cis* coupling constant and J_{BX} the *trans*, which is predicted by their dihedral angles. (However, if secondary amines were added to maleimides, "apparently simple spectra" resulted, with $|J_{AX} + J_{BX}|$ approximately 14 cycles/sec.) J_{AB} was not determinable in such systems due to the overlap of signals. The products from the primary amines were also formed more slowly, and the reactions are not reversible by acid (Smyth *et al.*, 1964) while the products from the secondary amines were formed relatively rapidly, and the amine could be regenerated by acid or heat. The nmr spectra differences thus correlate with differences in both ease of formation and stability of the two groups of compounds.

The possibility of the reaction of amines with maleimides interfering with the determination of sulfhydryl group rests upon both steric and kinetic factors. Gregory (1955) investigated the reaction of *N*-ethylmaleimide with glutathione and Lee and Samuels (1964) have investigated its reaction with L-cysteine hydrochloride. An estimate of the relative rates from these authors' data indicate that at about pH 7 the reaction of proline with *N*-ethylmaleimide is only about 10^{-3} that of either cysteine or glutathione.

On the other hand myokinase (Gregory, 1955) is much less rapid than glutathione, and thus reactivity of various enzymes may depend on the availability of sulfhydryl or amino reactive sites. There is little to suggest that maleimides are effective acylating agents. To our knowledge the only acylation reaction that has been reported for these compounds is the base-catalyzed solvolysis. A more characteristic, if not unique, property is the presence of a very strongly electrophilic double bond in a compound which is soluble, and relatively stable, in neutral aqueous solution.

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[3] The kinetic data are sufficient to indicate that the reaction is probably first order with respect to the maleimides. Presumably it is also first order with respect to the amine, but the preliminary data are insufficient to establish this unequivocally. Then, using the second-order kinetic equation, reaction rate constants can be estimated. The more rapid rate of *N*-ethylmaleimide over maleimide is borne out in the cases of both proline and piperidine at pH 8.5: proline, *N*-ethylmaleimide (C = 0.001) k_2 = 0.059 l. sec^{-1} $mole^{-1}$; proline, maleimide (C = 0.0015) k_2 = 0.020 l. sec^{-1} $mole^{-1}$; proline, maleimide (C = 0.002) k_2 = 0.019 l. sec^{-1} $mole^{-1}$; piperidine, *N*-ethylmaleimide (C = 0.001) k_2 = 0.10 l. sec^{-1} $mole^{-1}$; piperidine, maleimide (C = 0.0015) k_2 = 0.022 l. sec^{-1} $mole^{-1}$.

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Sedimentation Equilibrium in Reacting Systems. IV. Verification of the Theory*

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ABSTRACT: The theories [Adams, E. T., Jr., and Williams, J. W. (1964), *J. Am. Chem. Soc. 86*, 3454; Adams, E. T., Jr., (1965), *Biochemistry 4*, 1646] previously developed for nonideal associating systems have been verified by synthetic examples. The effects of variation in the virial coefficients or equilibrium constants on the association have been described. Details of the methods for evaluating $M_1/M_{n\,app}$ and $c_1e^{BM_1c}$ from the experimental data have been described.

The data of Rao and Kegeles [Rao, M. S. N., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724] on α-chymotrypsin have been examined, and it has been shown that one can do the analysis without recourse to additional sedimentation velocity experiments and the subsequent application of the Gilbert theory [Gilbert, G. A. (1955), *Discussions Faraday Soc. 20*, 68; Gilbert, G. A. (1963), *Ultracentrifugal Anal. Theory Expt. Conf. Rockefeller Inst. 1962*, 73; Gilbert, G. A., and Jenkins, R. C. L. (1963), *Ultracentrifugal Anal. Theory Expt. Conf. Rockefeller Inst. 1962*, 59; Nichol, L. Bethune, J. L., Kegeles, G., and Hess, E. L. (1964), *Proteins 2*, 305]. An analysis of some sedimentation equilibrium data on lysozyme at 15 and 25° and at pH 6.7 (20°) is reported. At both temperatures the lysozyme appears to undergo a monomer–dimer association; the association is more pronounced at 15°. A discussion of sources of experimental error is also included.

There are many molecules (soaps, detergents, some proteins, some chelate compounds, etc.) that undergo in solution reversible association reactions of the types

$$nP_1 \rightleftharpoons P_n, \qquad n = 2, 3, \ldots \tag{1}$$

or

$$nP_1 \rightleftharpoons qP_2 + mP_3 + \ldots \tag{2}$$

Here P represents a molecule, generally a macromolecule, undergoing the association–dissociation reaction. From sedimentation equilibrium experiments on these chemically reacting systems the quantities c (concentration) and $M_{w\,app}$ are obtained. In ideal solutions $M_{w\,app}$[1] (the apparent weight molecular weight) becomes $M_{w(c)}$ (the weight-average molecular weight).

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[1] The quantity $M_{w\,app}$ is the apparent weight-average molecular weight at any radial position, r, in the ultracentrifuge cell between the meniscus position, $r = a$, and the cell bottom position, $r = b$. $M_{w\,app}$ is given by the equation $d \ln c/d(r^2) = AM_{w\,app}$. Here, $A = (1 - \bar{v})\omega^2/2RT$; $\bar{v}$ = partial specific volume of the associating solute. (It is assumed that $\bar{v}_{Monomer} = \bar{v}_{Dimer} = \bar{v}$, *i.e.*, all partial specific volumes of the associating species are equal.) ρ = the density of the solution; ω = the angular velocity ($\omega = 2\pi$ rps); R = the gas constant; T = the absolute temperature; $c = c_r$ = the total concentration of the associating macromolecule in the solution column of the ultracentrifuge cell at any radial position between the meniscus ($r = a$) and the cell bottom ($r = b$).