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ACYLATION REACTIONS WITH CYCLIC IMIDES

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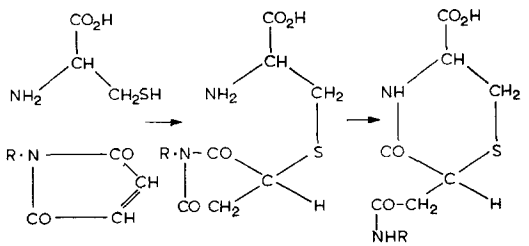
SUMMARY

Maleimide reagents have been examined for a potential use in the cross-linking of amino and thiol groups in proteins. The adducts obtained by reaction of *N*-ethylmaleimide or of *N*-(4-dimethyl-3,5-dinitroaminophenyl)maleimide with cysteine, homocysteine, and glutathione were prepared and the rates of reaction of the imide rings with water and with amino groups were studied. In the cysteine–maleimide addition products, where amino and thiol groups are located in positions sterically favourable for cross-linking, intramolecular aminolysis occurs readily. In contrast, the amino group of the homocysteine and glutathione adducts is comparatively stable.

Acylation reactions with cyclic imides

Previous studies on the reactions of *N*-ethylmaleimide with cysteine[1], and of *N*-(4-dimethyl-3,5-dinitroaminophenyl)maleimide (DDPM) with cysteine or with proteins containing thiol and amino groups[2], have shown that the reaction products can undergo intramolecular transamidation to form thiazine derivatives. A similar intramolecular acylation has been postulated in experiments on the reaction of



N-ethylmaleimide with hemoglobin[3]. The present study is concerned with investigating the steric conditions that are compatible with the use of maleimide reagents for the intramolecular cross-linking of thiol and amino groups in model compounds. If the relative positioning of the two groups should prove to be critical for cross-linking, the reagents could prove valuable in investigations of protein conformation; certain

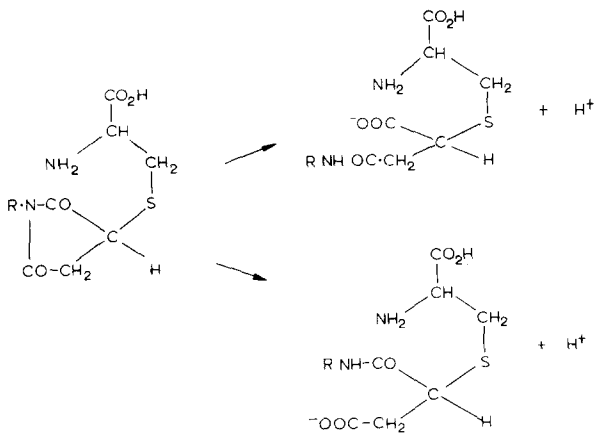
Abbreviation: DDPM, *N*-(4-dimethyl-3,5-dinitroaminophenyl)maleimide.

cysteine and lysine residues far apart in the linear sequence of protein could be identified as being closely positioned in space.

To examine this possibility, we have isolated the products obtained by addition of *N*-ethylmaleimide or of DDPM to the thiol group of cysteine, homocysteine, and glutathione and we have investigated the degree to which the derivatives undergo intramolecular aminolysis. Such transamidation reactions would lead to the formation of 6, 7 and 11 membered rings, respectively. In addition, the potential capacity of the maleimide–thiol adducts to act as intermolecular acylating agents has been examined by studying the reactivity of the imide ring of the addition product of *N*-ethylmaleimide with thioglycolic acid, and of DDPM with *N*-acetyl-L-cysteine, towards the amino group of triglycine.

RESULTS AND DISCUSSION

When a maleimide adduct of cysteine, homocysteine, or glutathione is maintained in neutral or alkaline solution, three competing reactions involving the imide ring may take place: hydrolysis, intramolecular aminolysis, and intermolecular aminolysis. Hydrolysis of the imide ring results in a release of H^+ by ionisation of the formed carboxyl group; provided no change occurs in the pK of the associated amino group, one H^+ is released corresponding to each imide ring undergoing hydrolysis. Aminolytic



reactions, both intra- and intermolecular, take place between imide carbonyl groups and nucleophilic amino groups. This reaction leads to a displacement of the equilibrium between charged and uncharged amino groups and may be accompanied by a release of H^+; the extent to which H^+ is liberated depends on the pH of the solution and the pK of the $-NH_3^+$ group.

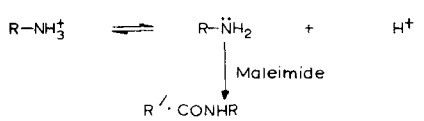


TABLE I

DECOMPOSITION OF MALEIMIDE–THIOL ADDITION PRODUCTS

3 mM solutions were titrated at constant pH with 0.1 M NaOH. Experiments were observed at 30.5° for at least 3 times the relevant half life period. Each recorded result is the mean of experiments performed in triplicate in which the greatest observed deviation was 5%.

Substance	*pH*	$K \times 10^3$ *(min^{-1})*	$t_{\frac{1}{2}}$ *(min)*
N-Ethylmaleimide	8.5	27	25.3
Cysteine–*N*-ethylmaleimide adduct	8.5	5.6	125
Cysteine–*N*-ethylmaleimide adduct	9.5	26	27.0
Homocysteine–*N*-ethylmaleimide adduct	9.5	4.1	168
Glutathione–*N*-ethylmaleimide adduct	9.5	4.0	176
N-Ethylmaleimide–thioglycolic acid adduct	8.5	0.22	3230
N-Ethylmaleimide–thioglycolic acid adduct	9.5	2.3	301
N-Ethylmaleimide–thioglycolic acid adduct + triglycine (1:1)	9.5	2.8	248
DDPM	7.0	18.7	37.2
Cysteine–DDPM adduct	7.0	43.1	16.2
Homocysteine–DDPM adduct	7.0	2.74	253
Glutathione–DDPM adduct	7.0	2.8	249
DDPM–*N*-acetylocysteine adduct	7.0	1.04	671
DDPM–*N*-acetylocysteine adduct + triglycine (1:1)	7.0	1.2	578

The reactions undergone by the eight maleimide adducts were studied with the aid of a pH stat (Radiometer Inc. Model TTT1a), which allowed measurement of the rates of formation of H^+. Dilute NaOH was added automatically to maintain the pH at the required value and the rates of addition were recorded graphically. From the

TABLE II

CHROMATOGRAPHY OF MALEIMIDE–THIOL ADDITION PRODUCTS AFTER INCUBATION AT ALKALINE pH VALUES

Reaction mixtures were analysed by paper chromatography, as described in EXPERIMENTAL.

Reactants	R_F *of reactant*	R_F *values of ninhydrin-positive products*	R_F *values of sulfur-containing product*
Thioglycolic acid–*N*-ethylmaleimide adduct (pH 9.5)	0.84	—	0.74
Thioglycolic acid–*N*-ethylmaleimide adduct + triglycine (1:1) (pH 9.5)	0.84		0.74
	0.10	0.10	0.21
Cysteine–*N*-ethylmaleimide adduct (pH 8.5)	0.27	—	0.58
Cysteine–*N*-ethylmaleimide adduct (pH 9.5)	0.27	0.16	0.58
			0.16
Homocysteine–*N*-ethylmaleimide adduct (pH 9.5)	0.41	0.30	0.70
		0.16	0.30
			0.16
Glutathione–*N*-ethylmaleimide adduct (pH 9.5)	0.27	0.17	0.18
			0.16

curves obtained it was found that first-order kinetics were obeyed in all cases, the slope of the first-order plots permitting calculation of the reaction constants given in Table I. In addition, the composition of the reaction mixture obtained on incubation of each *N*-ethylmaleimide adduct was examined by paper chromatography, and these results are indicated in Table II.

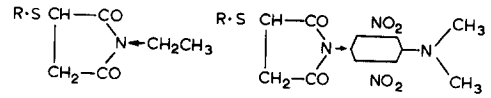
The addition product of cysteine with *N*-ethylmaleimide at pH 8.5, 30.5°, was found to undergo a first-order reaction ($t_{\frac{1}{2}}$ = 125 min resulting in total disappearance of amino groups; aminolysis appeared to occur exclusively with the formation of the thiazine derivative (R_F 0.58). At pH 9.5, on the other hand, the intramolecular re-arrangement was accompanied by some hydrolysis, and chromatography permitted the detection of a small amount of the ninhydrin-positive hydrolysis product (R_F 0.16) considered to be *S*-[(1-carboxy-2-*N*-ethylcarbamoyl)ethyl]-L-cysteine: $HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot CH(CO_2H) \cdot CH_2CONHC_2H_5$. The addition product of cysteine with DDPM exhibited a half life period of 16 min at pH 7.0. Chromatography of the reaction mixture showed the presence of a single, ninhydrin-negative compound; no hydrolytic product was observed. The thiazine derivatives formed in these re-arrangements have been previously isolated and characterized[1,2].

The addition product of *N*-ethylmaleimide with homocysteine underwent a slow, first-order reaction at pH 9.5 ($t_{\frac{1}{2}}$ = 168 min). Chromatography of the reaction mixture showed a ninhydrin-positive hydrolysis product (R_F 0.30) which was considered to be *S*-[(1-carboxy-2-*N*-ethylcarbamyl)ethyl]homocysteine. A very small amount of a ninhydrin-negative compound (R_F 0.70), possibly a thiazine homologue, was also formed; in addition there was a trace of a second ninhydrin-positive product (R_F 0.16), probably formed by intermolecular aminolysis. Analogous results were obtained with the DDPM–homocysteine addition product at pH 7.0 ($t_{\frac{1}{2}}$ = 253 min).

The addition products of glutathione with *N*-ethylmaleimide and with DDPM, at pH values 9.5 and 7.0, respectively, underwent slow hydrolysis. Chromatography of the reaction mixture of the glutathione–*N*-ethylmaleimide addition product did not reveal a ninhydrin-negative component or glutamic acid, which would have been released had intramolecular aminolysis occurred. A very small amount of a secondary product (R_F 0.18), possibly formed by intermolecular aminolysis, was noted.

The addition product of *N*-ethylmaleimide with thioglycolic acid at pH 9.5 underwent slow hydrolysis ($t_{\frac{1}{2}}$ = 301 min). Addition of triglycine in equimolar concentration caused an increase in the rate of decomposition and chromatography of the reaction mixture revealed, in addition to the main product (R_F 0.74) formed by hydrolysis, a small amount of ninhydrin-negative, sulfur containing compound (R_F 0.21), different from triglycine (R_F 0.10). Elution of this product from the chromatogram, followed by hydrolysis at 100° for 12 h in 6 M HCl, yielded only the free amino acid glycine. These observations are consistent with the occurrence of intermolecular aminolysis between triglycine and the imide ring of the maleimide–thioglycolic acid adduct.

From the results recorded in Table I, it is seen that the imide ring of the *N*-ethylmaleimide adducts exhibit striking differences in reactivity from the imides derived from DDPM. This effect is no doubt related to the different inductive effects of the group attached to the imide nitrogen atom, the dinitrodimethylaminophenyl group being electron attracting and the ethyl group electron donating.



N-ethylmaleimide adduct

DDPM adduct

In the series of reactions involving *N*-ethylmaleimide adducts, a comparison between the observed liberation of H^+ and the value calculated on the one hand for 100% aminolysis or on the other for 100% hydrolysis has enabled an assessment to be made of the relative contribution of each process to the overall reaction. The accuracy of the conclusions based on such an analysis depends on the degree of error involved in the measurement of the amino p*K* value and on the assumption that no change occurs in this value as a result of hydrolysis of the imide ring. The results obtained (Table III), interpreted within these limitations, confirm that intramolecular aminolysis is a reaction undergone readily only by the cysteine–*N*-ethylmaleimide adduct.

From these experiments the following general conclusions can be drawn; intramolecular cross-linking of amino and thiol groups by maleimide reagents occurs only

TABLE III

COMPARISON OF AMINOLYTIC AND HYDROLYTIC REACTIONS EXHIBITED BY *N*-ETHYLMALEIMIDE–THIOL ADDITION COMPOUNDS

Reactants were incubated at pH 9.5; p*K* values were measured by potentiometric titration.

Reactants	*pK of* $-NH_3^+$ *group*	*Theoretical number of* H^+ *formed from 100 molecules of reactant*		
		By hydrolysis	*By aminolysis*	*Observed*
Thioglycolic acid–*N*-ethylmaleimide adduct + triglycine (1:1)	7.9	100	2.5	97
Cysteine–*N*-ethylmaleimide adduct	8.8	100	16.6	15
Homocysteine–*N*-ethylmaleimide adduct	9.0	100	23.7	88
Glutathione–*N*-ethylmaleimide adduct	9.15	100	30.7	95

when the groups are located in sterically favourable positions, and under optimal conditions the competing reactions of intermolecular aminolysis and hydrolysis are minor; DDPM adducts undergo both aminolysis and hydrolysis more readily than *N*-ethylmaleimide adducts; saturation of the olefinic double bond of *N*-ethylmaleimide or DDPM by addition of a thiol compound greatly increases the stability of the imide ring.

EXPERIMENTAL

All melting points were uncorrected.

Materials

N-Ethylmaleimide (m.p. 45°) was obtained from Sigma Chemical Corporation.

DDPM was prepared according to the method of WITTER AND TUPPY[2]. Cysteine hydrochloride monohydrate was obtained from Sigma Chemical Company, DL-homocysteine from Nutritional Biochemicals Corporation, glutathione from Eastman Kodak Company, thioglycolic acid from Fisher Scientific Company, and glycylglycylglycine from Mann Research Laboratories Inc. *N*-Acetyl-L-cysteine was obtained as a generous gift from Dr. HANS T. CLARKE. The addition product of *N*-ethylmaleimide with L-cysteine, 5-(1-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine, was prepared as described previously[1,2]. The addition product of *N*-ethylmaleimide with thioglycolic acid was prepared according to the method of MARRIAN[4]. The addition product of DDPM with L-cysteine was prepared as described by WITTER AND TUPPY[2].

Chromatography

The reaction products in samples of reaction mixtures were separated by ascending filter paper chromatography on Whatman No. 1 paper using butanol–acetic acid–water (4:1:5, v/v/v, upper layer) as the solvent. The running time was about 12 h at room temperature. Chromatograms were dried in a current of air without the application of heat, then were sprayed with a 0.1% (w/v) solution of ninhydrin in acetone or with an aqueous potassium iodide–chloroplatinic acid solution prepared as described by WINEGARD, TOENNIES AND BLOCK[5], and were again allowed to dry at room temperature. Chromatography of reaction mixtures containing DDPM–thiol addition products was facilitated by the intense yellow colour exhibited by these compounds.

S-(1-Ethyl-2,5-dioxopyrrolidin-3-yl)-DL-homocysteine

DL-Homocysteine (135 mg, 1 mmole) was dissolved in water (10 ml), initial pH 6.1, *N*-ethylmaleimide (125 mg, 1 mmole) was added and the flask was gently agitated to effect solution. After 1 h, acetone (20 ml) was added and a pale yellow solid (207 mg, m.p. 298–299° decomp.) which separated was filtered off and dried in a current of air. Recrystallisation of this material from an aqueous solution (4 ml) by cautious addition of acetone (20 ml) gave 196 mg of a white, crystalline product (m.p. 214° decomp.). A second crystallisation using the same conditions gave 171 mg of a product with the same melting point.

Analysis: Calculated for $C_{10}H_{16}N_2O_4S$: C, 46.1; H, 6.2; N, 10.8%. Found: C, 46.0; 6.3; N, 10.8%.

S-(1-Ethyl-2,5-dioxopyrrolidin-3-yl)-glutathione

Glutathione (307 mg, 1 mmole) was dissolved in water (10 ml) and the solution was neutralised to pH 6.0 by addition of 10 ml of 0.1 M aq. NaOH. A solution of *N*-ethylmaleimide (125 mg, 1 mmole) in water (10 ml) was added, and the solution was allowed to stand at room temperature for 3 h. The resulting solution was titrated with 10 ml of 0.1 M HCl, then was evaporated *in vacuo* at 35° to about 5 ml. Addition of acetone (20 ml) precipitated a white crystalline solid (438 mg, m.p. 205° decomp.) which was filtered off and dried. Recrystallisation from water (10 ml) by addition of acetone (30 ml) gave the pure product (383 mg, m.p. 206° decomp.).

Analysis: Calculated for $C_{16}H_{24}N_4O_8S$: C, 44.4; H, 5.6; N, 12.9%. Found: C, 44.4; H, 5.7; N, 12.6%.

The addition product of DL-*homocysteine with DDPM*

DL-Homocysteine (135 mg, 1 mmole) was dissolved in 2 ml of water and was mixed with a solution of DDPM (206 mg) dissolved in 3 ml of acetone. The mixture was left at room temperature for 15 min during which time some crystalline material separated. Crystallisation was made more complete by addition of another 5 ml of acetone. The precipitate (154 mg) was filtered off and washed with acetone. From the filtrate, addition of ethyl ether and standing permitted the isolation of a further quantity (79 mg) of product. The combined products were suspended in water (4 ml) and were brought into solution by dropwise addition of 1 M HCl. To the acid solution a strong solution of sodium acetate was slowly added, drop by drop, with good stirring. The product separated out in finely crystalline form. Addition of sodium acetate was slowly continued until the supernatant fluid was pale yellow. The product was filtered off, and washed repeatedly with water (209 mg, m.p. indefinite). The purification procedure was repeated. 1 M HCl acid was added to a suspension of the yellow material in water until it was almost completely dissolved. The undissolved material was filtered off. To the filtrate, sodium acetate solution was added with scratching until the pH was 4.5. The yellow crystalline precipitate was filtered off, washed many times with water, followed by acetone and ether. The product (155 mg) was dried at 78° for 2 h *in vacuo*.

Analysis: Calculated for $C_{16}H_{19}O_8N_5S$: C, 43.5; H, 4.3; N, 15.9%. Found: C, 43.4; H, 4.6; N, 15.9%.

*The addition product of DDPM with N-acetyl-*L-*cysteine*

N-Acetyl-L-cysteine (163 mg, 1 mmole) was dissolved in 5 ml of acetone, and DDPM (206 mg, 1 mmole) dissolved in acetone (5 ml) was added. This solution was divided into two halves. One half was treated with ether, then with light petroleum and was allowed to stand for 12 h in a refrigerator. The material partially crystallised. When the precipitate was washed with ether containing acetone, some amorphous material (18 mg, m.p. 194–195°) was left behind. The wash fluids and filtrate were combined and brought to dryness in vacuo at 35°, dissolved in a small amount of acetone, and a small amount of ether was added until turbidity appeared; the solution was seeded and left to stand at room temperature. From time to time, more ether was added, and crystalline material (71 mg) was thus obtained. Recrystallisation was effected by dissolving in a small amount of hot acetone. After the solution had cooled, ether was slowly added in several portions at intervals of approximately 15 min. Most of the yellow colour was present in the crystalline product (63 mg) which after filtration, washing with ether, and drying at 78° for 2 h *in vacuo* had a m.p. of 192–193° decomb.

Analysis: Calculated for $C_{17}H_{19}N_5O_9S$: C, 43.5; H, 4.1; N, 14.9%. Found: C, 43.7; H, 4.2; N, 15.1%.

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