was found. Proceeding from there, a solution of 3.56 g. (0.010 mole) of carbobenzoxyglycyl-L-phenylalanine [having a melting point of 127.5–128.0°, and an optical rotation $[\alpha]^{25}D$ + 38.2° (*c* 5, abs. ethanol) compared with values[4] of m.p. 127.5° and $[\alpha]^{21}D$ + 38.8 ± 0.5° (*c* 5, abs. ethanol)] in 10 ml. of dried (calcium hydride) dimethylformamide was cooled to −10° and 1.65 g. (0.010 mole based on 98% purity) of N,N′-carbonyldiimidazole was added. When the slow effervescence stopped, 1.03 g. (0.010 mole) of freshly-distilled ethyl glycinate was added. The reaction solution was allowed to warm to room temperature and permitted to stand 15–30 min. Then 50 ml. of 1 *N* hydrochloric acid was added. When the oily liquid thus formed solidified, it was washed with 20 ml. of a 5% sodium bicarbonate solution and with water. On drying, 4.22 g. (96% yield) with a melting point of 115.5–117.0° was obtained. The product was dissolved in 210 ml. of absolute ethanol to give a 2% solution. After cooling to 0°, the solution was seeded with a crystal of ethyl carbobenzoxyglycyl-DL-phenylalanylglycinate. Fractions were cut as follows:

No.	Time, hr.	Wt., g.		M.p., °C.
1	3	0.0091		120.0–133.5
2	6	.0097		119.0–128.5
3	10	.0214		118.5–119.5
4	24	2.3201		119.8–120.1
	Concd.	1.4787		119.9–120.3
	Residue	0.3271		
	DL-Isomer	0.0188	0.45%	
	L-Isomer	3.8202	87%	$[\alpha]^{25}D$ −12.2 ± 1.25° (*c* 2, EtOH)
	Residue	0.3271		
	Material balance	4.1661		

Since the melting point of pure ethyl carbobenzoxyglycyl-DL-phenylalanylglycinate was reported[4] to be 132–133° and has been found in other reactions by us to be 133.0–133.5°, the percentage of DL-tripeptide is estimated from the melting points to be much less than 0.5%.

In other reactions, run in the same way, varying one or two factors, more racemization was found. Running the reaction in THF at room temperature gave 5% DL-tripeptide. Using ethyl glycinate hydrochloride at room temperature in THF gave 8% DL-tripeptide. Running the reaction in dimethylformamide at 0–5° gave 1.3% racemization.

In these studies, the melting point of the carbobenzoxyglycyl-L-phenylalanine used was very important since commercial L-phenylalanine may contain several per cent. of the DL-isomer.

Ethyl Carbobenzoxyglycyl-DL-phenylalaninate using N,N′-Carbonyldibenzimidazole.—A solution of 2.09 g. (0.010 mole) of carbobenzoxyglycine in 10 ml. of dry THF was treated with 2.62 g. (0.010 mole) of N,N′-carbonyldibenzimidazole.[5] When no effervescence was noted, and the reagent only partially dissolved, another 10 ml. of THF was added. Again nothing happened and the mixture was heated under reflux for 10 min. A solution formed and 1.93 g. (0.010 mole) of ethyl DL-phenylalaninate (freshly distilled) was added. After heating for 15–30 min. on a steam-bath, most of the solvent boiled off. A 50-ml. quantity of 1 *N* hydrochloric acid was then added. Cooling and scratching gave a solid product. This was washed first with water, then with 20 ml. of 5% sodium bicarbonate solution and finally with water again. The crude product weighed 3.48 g. (77% yield) and had a melting point range of 83.5–88.0°. Recrystallization from 20 ml. of ethyl acetate and 40 ml. of petroleum ether resulted in 2.75 g. of compound with a melting range of 85–88°. This material was recrystallized again, this time from 20 ml. of benzene and 40 ml. of petroleum ether, giving 2.63 g. (69% yield) of ethyl carbobenzoxyglycyl-DL-phenylalaninate with a melting point of 89.5–91.0° as compared to a previously cited melting point of 90–91°. Because of the poorer yield and the more rigorous conditions necessary for a reaction, N,N′-carbonyldibenzimidazole is considered inferior to N,N′-carbonyldiimidazole.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, YALE UNIVERSITY]

Some Reactions of N-Ethylmaleimide[1]

BY DEREK G. SMYTH,[2] ATSUO NAGAMATSU[3] AND JOSEPH S. FRUTON

RECEIVED MARCH 7, 1960

The reactions of N-ethylmaleimide (NEM) with the amino group of peptides, with imidazole and with cysteine have been investigated. With the first two classes of compound, an N-acylation reaction appears to occur, followed in the case of imidazole by a catalytic polymerization of NEM. With cysteine, reaction proceeds through addition of the thiol to the olefinic bond of NEM; in alkaline solution, the cysteine adduct undergoes an intramolecular transamidation reaction to form a thiazane derivative.

During the course of studies on the action of cysteine-activated cathepsin C[4] on glycyl-L-histidinamide at *p*H 7.4, N-ethylmaleimide (NEM) was used to facilitate chromatographic examination of the composition of the incubation mixture. The reaction of NEM with sulfhydryl compounds[5,6] had been used by Hanes, *et al.*,[7] to stabilize glutathione and other sulfhydryl peptides in paper chromatography. When the NEM-treated incubation mixtures of cathepsin C and glycyl-L-histidinamide were chromatographed, a number of Pauly-reactive components of widely different R_f values were observed.[8] Subsequent control experiments demonstrated, however, that the new products (other than the expected hydrolytic product, glycyl-L-histidine, or the unchanged dipeptide amide) arose in incubation mixtures to which no enzyme had been added. Further investigations showed that one of the new Pauly-positive products was noted only when NEM had been used before paper chromatography, and led to the recognition that NEM is not specific toward sulfhydryl compounds, as had previously been supposed. In the present communication we report some reactions of NEM with imidazole and its derivatives, with the α-amino group of

(1) This investigation was supported by grants from the National Science Foundation (G-7451) and the United States Public Health Service (RG-6452).

(2) Alexander Brown Coxe Fellow of the Yale School of Medicine, 1959–1960.

(3) James Hudson Brown Fellow of the Yale School of Medicine, 1958–1959. On leave from the Department of Biochemistry, Kyushu University, Fukuoka, Japan.

(4) N. Izumiya and J. S. Fruton, *J. Biol. Chem.*, **218**, 59 (1956).

(5) E. Friedmann, D. H. Marrian and I. Simon-Reuss, *Brit. J. Pharmacol.*, **4**, 105 (1949); *Biochim. Biophys. Acta*, **9**, 61 (1952).

(6) D. H. Marrian, *J. Chem. Soc.*, 1515 (1949).

(7) C. S. Hanes, F. J. R. Hird and F. A. Isherwood, *Biochem. J.*, **51**, 25 (1952).

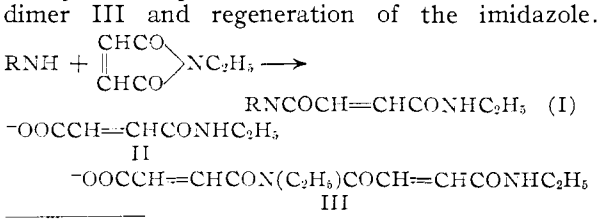
(8) J. Barnabas, unpublished experiments.

peptides and with the sulfhydryl group of cysteine. The results obtained suggest the need for caution in the use of NEM for the detection of sulfhydryl compounds in mixtures derived from biological sources or formed in enzymic experiments.

Reaction of NEM with Imidazole.—When 0.2 *M* imidazole and 0.2 *M* NEM were allowed to react in water (initial *p*H 6.4), a pink color appeared within 2 minutes, and a crystalline precipitate separated. Chromatographic examination of the supernatant fluid showed the presence of only one Pauly-positive (yellow) spot, whose R_f was identical with that of imidazole. The crystals had a high melting point, and dissolved in alkaline solutions (above *p*H 6) with the appearance of a dark red color. At very alkaline *p*H values, the color slowly disappeared. Upon acidification, the solution was decolorized, and the substance crystallized. After paper chromatography, the product gave a single red spot (R_f 0.95) upon treatment with alkaline carbonate; no Pauly-positive yellow spot was observed. The material appeared to have a molecular weight of about 550. A possible structure of the polymeric product is N-ethyl-(N-ethylmaleamyl)$_n$-maleamic acid; this structure has been proposed previously[9] for a by-product in the synthesis of N-alkylmaleimides by the reaction of aliphatic amines with maleic anhydride at 170°. It does not possess, however, the extended conjugated unsaturation to be expected of a substance exhibiting color in alkaline solution. Further work is needed, therefore, to establish firmly the structure of the NEM polymer formed in the presence of imidazole.

That imidazole acts catalytically in the formation of the crystalline product was shown by the isolation of the latter in 50% of the theory (based on NEM) when an aqueous solution of 0.2 *M* NEM and 0.02 *M* imidazole was kept at room temperature for 5 days (initial *p*H about 6). When 0.1 *M* NEM was incubated at room temperature for 5 days in 0.1 *M* phosphate buffer (*p*H 7.4), a spot corresponding to N-ethylmaleamic acid (R_f 0.75) was observed.

A plausible explanation of the phenomena described above is that the imidazole (RNH) attacks one of the 2 CO groups of NEM to form an acylimidazole (I) which may be expected to undergo rapid hydrolysis to N-ethylmaleamic acid (II). The latter component has been shown to arise by the slow hydrolysis of NEM at *p*H values more alkaline than 7.[10] Since acylimidazoles are reactive acylating agents,[11] compound I may be expected to acylate compound II with the formation of the dimer III and regeneration of the imidazole.



(I)

II

III

(9) L. E. Colemann, J. F. Bork and H. Dunn, *J. Org. Chem.*, **24**, 135 (1959).

(10) J. D. Gregory, THIS JOURNAL, **77**, 3922 (1955).

(11) E. R. Stadtman, *ibid.*, **75**, 2022 (1953).

Compound III would be susceptible to further acylation by I, and the chain length of the resulting polymer would thus increase until a sparingly soluble product was formed. In accord with this explanation is the observation that the addition of N-ethylmaleamic acid to the buffered solution of NEM and imidazole markedly reduced the rate of polymer formation.

It is noteworthy that the properties of the solution containing imidazole and NEM were identical with those reported by Benesch, *et al.*,[12] for the reaction of NEM with thiols. Application of the Benesch test, previously considered specific for thiols and thiol esters, to imidazole and imidazole derivatives gave dark red colors. Also, it should be added that the appearance of dark red colors upon treatment of N-alkylmaleimides with alkali had been reported in the pioneer work of Piutti on these compounds.[13]

Reaction of NEM with Histidine Derivatives.—When in place of imidazole, histidine derivatives (0.25 *M*) were tested at *p*H 7.4 for their ability to produce a pink color and an insoluble NEM polymer, positive results were obtained in all cases. With α-N-acetyl-DL-histidine, carbobenzoxyglycyl-L-histidinamide or L-histidine anhydride, chromatographic examination of the reaction mixture showed only one yellow spot with the Pauly reagent; the R_f in each case was that of the unchanged histidine derivative. With glycyl-L-histidinamide, however, there gradually appeared a new Pauly-positive spot (Table I), with the progressive disappearance of the original dipeptide amide. In the solvent system used for chromatography, this new component moved more rapidly than the starting material, and was ninhydrin-negative when the chromatogram was developed at room temperature. When the ninhydrin-treated chromatogram was heated at 100°, a positive reaction was observed for this faster component. In this connection it should be noted that N-ethylmaleamic

TABLE I

CHROMATOGRAPHY OF COMPOUNDS INCUBATED WITH N-ETHYLMALEIMIDE[a]

Substance	R_f	Disappearance of reactant	R_f of product	Polymer formation
Glycyl-L-histidinamide	0.22	+	0.52	+
L-Histidine	.18	+	0.48	+
Carbobenzoxyglycyl-L-histidinamide	.77	−		+
α-N-Acetyl-DL-histidine	.26	−		+
L-Histidine anhydride	.33	−		+
Imidazole	.54	−		+
L-Leucine	.58	−		−
L-Leucinamide	.63	+		−
L-Leucyl-L-leucine	.84	+		−
L-Tyrosyl-L-tyrosine	.65	+	0.71	−
L-Cystine	.12	+	0.27	−

[a] 50 micromoles of each substance was incubated at room temperature with excess NEM (100 micromoles) for 6 hours in 2 ml. of 0.1 *M* phosphate buffer (*p*H 7.4).

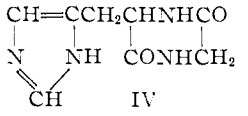
(12) R. Benesch, R. E. Benesch, M. Gutcho and L. Laufer, *Science*, **123**, 981 (1956).

(13) A. Piutti, *Ber.*, **39**, 2766 (1906).

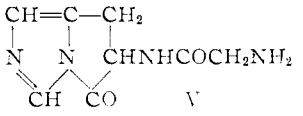
acid (II) and the polymeric product derived from NEM in the presence of imidazole at *p*H 6 also gave a positive ninhydrin reaction on chromatograms heated at 100°, but not when the paper was dried at room temperature. It may be concluded, therefore, that the faster Pauly-positive and ninhydrin-positive (at 100°) component observed in the reaction between NEM and glycyl-L-histidinamide is the result of the acylation of the α-amino group of the peptide by a N-ethylmaleamyl group.

The pK' of the α-amino group of glycylhistidinamide may be assumed to be near *p*H 8, and it appears likely that at *p*H 7.4 a sufficient fraction of the α-NH_3^+ groups have been converted to the nucleophilic α-NH_2 groups. However, a catalytic role of the imidazolyl group also appears likely. As will be seen from Table I, L-histidine (pK_3' 9.1) also reacted at *p*H 7.4 with NEM to yield a faster-moving Pauly-positive chromatographic component, with the gradual disappearance of the free amino acid. On the other hand, L-leucine (pK_2' 9.6) gave no evidence of a reaction with NEM at *p*H 7.4. It seems possible, therefore, that the imidazolyl group of histidine may exert a catalytic effect on acylation of the α-amino group by the intermediate formation of a reactive N-(N-ethylmaleamyl)-imidazolylalanine, followed by the transfer of the N-ethylmaleamyl group to the α-amino group.

During the course of these experiments, it was observed that, at *p*H 7.4, in the absence of NEM, glycyl-L-histidinamide gave rise to a chromatographic component (R_f 0.35) which was unreactive to ninhydrin at 100° and gave a yellow color with the Pauly reagent. Although this product was not isolated, it appears reasonable to assume that it is glycyl-L-histidine anhydride (IV), and that its formation involves the acylimidazole V as a possible intermediate. The synthesis and reactivity of intramolecular acylimidazoles such as V has recently been reported.[14]



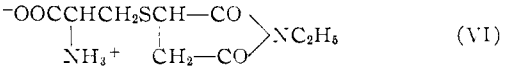
IV



V

Reaction of NEM with Amino Groups.—In view of the results summarized above, it was of interest to examine the reactivity of NEM toward the α-amino group of peptides and amino acid derivatives containing no imidazolyl group. When 0.25 *M* L-leucyl-L-leucine, L-leucinamide or L-tyrosyl-L-tyrosine were incubated with NEM at *p*H 7.4, chromatographic examination of the mixture showed the progressive disappearance of the ninhydrin-reactive component. In the case of tyrosyl-tyrosine, the formation of a new Pauly-positive product of slightly higher R_f could be demonstrated. It appears reasonable to conclude, therefore, that in all these cases acylation occurred at the α-amino group, with the formation of a N-(N-ethylmaleamyl) peptide. Since the pK' of the $-NH_3^+$ group of these compounds is near 8, a sufficient fraction of unprotonated α-NH_2 groups appears to be available for nucleophilic attack of the NEM molecule. In this connection, it is of interest that L-cystine, which has one NH_3^+ group of pK' near 8, also reacted with NEM at *p*H 7.4 to give a new chromatographic component identified by the ninhydrin reaction, presumably given by the NH_3^+ group of higher pK'. Since acylamino compounds resulting from the reaction of NEM with an α-amino group are stable, in contrast to acylimidazoles, no formation of the polymer derived from NEM was to be expected, and none was observed.

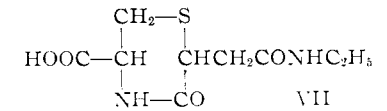
Reaction of NEM with Cysteine.—It is well known that the reaction of NEM with thiol compounds is very rapid as compared to hydrolysis at *p*H values near 7[10] and it is generally assumed that the reaction of cysteine involves addition of the SH group to the olefinic double bond of NEM to form the thioether VI.



(VI)

The isolation and characterization of this product as S-(N-ethylsuccinimido)-L-cysteine has not been reported previously, although the corresponding derivative of thioglycolic acid has been described,[6] as has been the adduct of cysteine and maleic acid.[15] The isolation of VI was readily effected in 84% yield from an aqueous reaction mixture (initial *p*H 6) containing 0.2 *M* L-cysteine and 0.2 *M* NEM. The resulting product was soluble in water (*p*H of aqueous solution, 6.0); it reacted with ninhydrin but gave a negative nitroprusside test for SH groups under the usual reaction conditions. It readily reacted at *p*H 9 with NH_2OH to form a hydroxamic acid, indicating the presence of the imide group. Potentiometric titration (Beckman model G *p*H meter) of a 0.1 *M* solution indicated the presence of two dissociating groups, having pK' values of about 2.2 and 8.8, which may be assigned to the α-carboxyl and α-ammonium groups of VI.

When a 0.1 *M* solution of VI is kept at room temperature and *p*H 9 for 36 hours, it undergoes nearly quantitative conversion into the cyclic compound VII (2-(N-ethylacetamido)-3-keto-1,4-thiazane-5-carboxylic acid), by an intramolecular transamidation reaction involving the attack of the α-NH_2 group on the imide CO group.[16] The isolation and characterization of compound VII



VII

serves as additional evidence for the formulation of the cysteine adduct of NEM as VI. The ready formation of the thiazane derivative at *p*H 9 provides further indication of the reactivity of the imide ring of NEM derivatives with amino groups. It also leads one to expect that attempts to retrace

(14) J. C. Sheehan, K. Hasspacher and Y. Lieh-Yeh, THIS JOURNAL, **81**, 6086 (1959).

(15) E. J. Morgan and E. Friedmann, *Biochem. J.*, **32**, 733 (1938).

(16) During the preparation of this manuscript, Prof. Hans Tuppy kindly informed us that experiments in his laboratory (A. Witter and H. Tuppy, unpublished) have shown that the cysteine adduct of N-(4-dimethylamino-3,5-dinitrophenyl)-maleimide, upon exposure to mild alkali, is transformed into the thiazane derivative corresponding to VII.

the titration curve of VI from *p*H 10 to *p*H 6 should show a decrease in the buffering capacity at *p*H 8.8; this was in fact observed.

Upon treatment of compound VI with *N* NaOH, the solution gives a positive nitroprusside test for SH groups. This may be a consequence of a cleavage similar to that undergone by β-ketothioethers in alkaline solution.[17]

Cysteine, like imidazole, promotes a polymerization of NEM at *p*H 7.4 and room temperature. On incubation of 0.02 *M* cysteine with 0.2 *M* NEM under these conditions, there appeared a pink crystalline precipitate (yield 12.5% based on NEM) whose properties were similar to those described above for the NEM polymer obtained in the presence of imidazole. Chromatographic examination of such a cysteine–NEM solution at *p*H 7.4 showed that the ninhydrin-reactive compound VI gradually disappeared as the polymer separated.

In view of the above findings, it is not surprising that under the conditions of the Benesch test, involving the use of alkali and excess NEM, compound VI gives a dark red color, associated with the formation of NEM polymer.

Experimental[18]

Materials.—2-N-Ethylmaleimide (m.p. 45°) was obtained from the Sigma Chemical Corp. N-Ethylmaleamic acid (m.p. 123–124°) was obtained by the hydrolysis of NEM with the calculated amount of dilute KOH, neutralization with dilute HCl, concentration under reduced pressure, and extraction of the product with absolute ethanol or acetone. Imidazole (m.p. 89°) and glutathione were obtained from Eastman Organic Chemicals. The following compounds were synthesized in this Laboratory according to published procedures: α-N-acetyl-DL-histidine,[19] L-histidine anhydride,[20] L-leucyl-L-leucine,[21] L-leucinamide acetate[22] and L-tyrosyl-L-tyrosine.[23]

Glycyl-L-histidinamide acetate was synthesized by the following procedure: A mixture of 4.18 g. of carbobenzoxyglycine, 2.8 ml. of triethylamine, 2.62 ml. of isobutyl chlorocarbonate and 50 ml. of toluene was chilled to −5° for 15 minutes, and then added to a chilled mixture of 4.84 g. of L-histidine methyl ester dihydrochloride, 5.6 ml. of triethylamine and 65 ml. of chloroform. The reaction mixture was kept at room temperature for 20 hours and concentrated *in vacuo* to a sirup, which was extracted with 30 ml. of ethyl acetate. The insoluble triethylamine hydrochloride was filtered off by suction, and the filtrate was concentrated *in vacuo* to yield a sirup (4.5 g.) which was treated with 100 ml. of methanol previously saturated at 0° with NH_3 gas. The mixture was allowed to stand at room temperature for 2 days, concentrated *in vacuo*, and the residual solid was triturated with absolute ethanol to yield 4.0 g. of the carbobenzoxy dipeptide amide, m.p. 207–208° dec.

Anal. Calcd. for $C_{16}H_{19}O_4N_5$: C, 55.6; H, 5.5; N, 20.3. Found: C, 55.7; H, 5.5; N, 20.5.

Upon hydrogenolysis (3 hours) of 4.14 g. of the carbobenzoxy compound in 70 ml. of methanol containing 0.8 ml. of glacial acetic acid, in the presence of Pd black, the dipeptide amide acetate separated from the solution. Water was added, the catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. Treatment of the resulting sirup with methanol–ethyl acetate gave 3.2 g. of the desired product. Upon paper chromatography, the compound showed only one component of R_f 0.21, either by means of the ninhydrin reagent or the Pauly reagent; $[\alpha]^{26}D$ + 14.9° (*c* 2 in H_2O).

Anal. Calcd. for $C_{10}H_{17}O_4N_5$: C, 44.3; H, 4.8; N, 25.8. Found: C, 44.6; H, 4.7; N, 26.1.

A similar procedure was used to synthesize L-alanyl-L-histidinamide acetate. From 2.23 g. of carbobenzoxy-L-alanine, 1.3 g. of carbobenzoxy-L-alanyl-L-histidinamide (m.p. 178–180°) was obtained.

Anal. Calcd. for $C_{17}H_{21}O_4N_5$: C, 56.8; H, 5.9; N, 19.5. Found: C, 56.8; H, 5.7; N, 19.7.

Hydrogenolysis in the manner described above gave the dipeptide amide acetate in theoretical yield. Upon paper chromatography, only one ninhydrin-reactive component (R_f 0.24) was observed; $[\alpha]^{24}D$ + 16.1° (*c* 2 in H_2O).

Anal. Calcd. for $C_{11}H_{19}O_4N_5$: C, 46.3; H, 6.7; N, 24.6. Found: C, 46.3; H, 6.9; N, 24.6.

Chromatography.—The reaction products in samples of reaction mixtures were separated by ascending paper chromatography on Whatman No. 1 paper, and with butanol–pyridine–acetic acid–water (30:20:6:24) as the solvent. The running time was normally about 9 hours at room temperature. Chromatograms were dried in a current of air without the application of heat, and then were sprayed with the appropriate agent. When a reactant contained an imidazolyl or a phenolic group, the chromatogram was sprayed with a butanol solution of Pauly reagent (prepared by mixing 5 ml. of 5% $NaNO_2$ with 5 ml. of 5% sulfanilic acid, and extracting the aqueous mixture with 20 ml. of 1-butanol); the paper was then sprayed with a half-saturated Na_2CO_3 solution in water. In other experiments, chromatograms were sprayed with a 0.1 % (w./v.) solution of ninhydrin in acetone, and were allowed to dry at room temperature.

Isolation of NEM Polymer.—Imidazole (680 mg., 10 mmoles) was dissolved in 25 ml. of water and the solution was mixed with a solution of 1.25 g. (10 mmoles) of NEM in 25 ml. of water. Within 2 minutes a pink color appeared and a precipitate separated. After 12 hours the precipitate was filtered and washed with water, giving 825 mg. of a product which was purified by crystallization from benzene; m.p. 195° dec. A solution of 80 mg. of the product in 4 ml. of acetic acid gave a freezing point depression of 0.15° (average of 3 determinations), corresponding to a molecular weight of approximately 550.

Anal. Found: C, 56.0; H, 5.6; N, 11.2.

The calculated values of N content for NEM polymers of increasing chain length do not differ greatly (*ca.* 11%), but the calculated value for C increases from 54.9 for the trimer to 56.8 for the decamer.

A second preparation of polymer was obtained from the reaction of 68 mg. (1 mmole) of imidazole and 1.25 g. (10 mmoles) of NEM dissolved in 50 ml. of water. During 5 days, repeated filtration permitted isolation of 622 mg. of product (50% yield based on NEM).

The polymer was soluble in alkali, forming an intense red solution from which no benzene-soluble material could be extracted. Acidification of the red solution produced a colorless solution from which the polymer rapidly separated and from which the polymer was easily extracted into benzene. Addition of petroleum ether (b.p. 30–60°) to the benzene solution precipitated the polymer as a white powder.

Effect of N-Ethylmaleamic Acid on the Polymerization Reaction.—Imidazole (6.8 mg., 0.1 mmole) and 14.5 mg. (0.1 mmole) of N-ethylmaleamic acid were dissolved in 1 ml. of 0.2 *M* phosphate buffer at *p*H 7.4, then 12.5 mg. (0.1 mmole) of NEM was dissolved in this solution. An identical control solution was prepared lacking N-ethylmaleamic acid. After 2 hours the first solution was homogeneous and exhibited a faint pink color, whereas the second solution was bright pink and on filtration yielded 4.5 mg. of polymer.

Chromatography of Thiols and Imidazoles Treated with NEM by the Benesch Technique.—Cysteine hydrochloride monohydrate (2 mg.) was dissolved in 0.5 ml. of 0.1 *M* phosphate buffer at *p*H 7.4 and 5 mg. of NEM (excess) was added. Analogous solutions were prepared from glutathione, imidazole and L-histidine hyrochloride monohydrate. The solutions were then kept for 12 hours at room temperature, then samples were chromatographed. The dried

(17) B. Nicolet, THIS JOURNAL, **53**, 3066 (1931).

(18) All melting points were uncorrected. Microanalyses were performed by Dr. S. M. Nagy, Department of Chemistry, Massachusetts Institute of Technology, to whom we wish to express our thanks.

(19) M. Bergmann and L. Zervas, *Biochem. Z.*, **203**, 280 (1928).

(20) E. Abderhalden and F. Leinert, *Fermentforschung*, **15**, 324 (1937).

(21) J. R. Vaughan and R. L. Osato, THIS JOURNAL, **74**, 676 (1952).

(22) O. K. Behrens and M. Bergmann, *J. Biol. Chem.*, **129**, 587 (1939).

(23) M. Bergmann, L. Zervas, L. Salzmann and H. Schleich, *Z. physiol. Chem.*, **224**, 17 (1934).

chromatogram was dipped in a 0.05 *M* solution of NEM in isopropyl alcohol, allowed to dry for 15 minutes, and then was dipped in a 0.25 *M* solution of potassium hydroxide in isopropyl alcohol. Each reactant gave rise to a single pink spot (R_f 0.95).

The procedure was repeated by incubating for 12 hours 17.5 mg. of L-cysteine hydrochloride monohydrate (100 μmoles), 30 mg. (100 μmoles) of glutathione, 7 mg. (100 μmoles) of imidazole and 19 mg. (100 μmoles) of L-histidine hydrochloride monohydrate separately with 12.5 mg. (100 μmoles) of NEM in 5 ml. of 0.1 *M* phosphate buffer at *p*H 7.4. With the thiols, reaction products (R_f 0.42 and 0.55, respectively) were observed; with the imidazoles, the pink reaction product (R_f 0.95) was observed.

Cysteine hydrochloride monohydrate (17.5 mg., 100 μmoles) was treated at room temperature for 7 days with 12.5 mg. (100 μmoles) of NEM in 5 ml. of 0.1 *M* phosphate buffer at *p*H 7.4 (solution 1). Solutions 2–5 were prepared using cysteine hydrochloride monohydrate (100 μmoles) and increasing amounts (200–500 μmoles) of NEM. After 7 days, solution 1 was homogeneous, and chromatography showed the exclusive presence of the product of R_f 0.42; solutions 2–5 showed increasing separation of pink crystals, and chromatography showed a progressive decrease in concentration of the product of R_f 0.42, and a simultaneous progressive increase in concentration of a second product of R_f 0.95. The first product was reactive to ninhydrin at room temperature, the second was reactive only after drying of the ninhydrin sprayed chromatogram at 100°.

S-(N-Ethylsuccinimido)-L-cysteine (VI).—To a solution of L-cysteine hydrochloride monohydrate (1.75 g., 10 mmoles) in 50 ml. of water was added 1.25 g. of NEM (10 mmoles), followed by 10 ml. of 1.0 *N* sodium hydroxide. The resulting solution (*p*H 5.8) was incubated for 30 minutes, and then was concentrated to 20 ml. under reduced pressure at 35°. On addition of 20 ml. of acetone, 2.1 g. (84%) of crystals separated and were washed with ethanol. Recrystallization from water–acetone yielded 1.2 g. of a product melting at 194–195° dec.

Anal. Calcd. for $C_9H_{14}O_4N_2S$: C, 43.9; H, 5.7; N, 11.4; S, 13.0. Found: C, 43.8; H, 5.7; N, 11.5; S, 13.2.

One mmole (246 mg.) of the product VI was dissolved in 10 ml. of water, and 12.5 ml. of 0.08 *N* potassium hydroxide was added in small portions over a period of 15 hours. The *p*H of the solution at no time exceeded 9.2. After 36 hours, 9.2 ml. of 1.08 *N* hydrochloric acid was added, and the solution was concentrated to dryness *in vacuo* at 35°; 5 ml. of water was added, and the distillation was repeated. The distillation was twice repeated after addition of 20 ml. of absolute ethanol. The residual white crystalline solid was dried, and then extracted 4 times with 20-ml. portions of anhydrous acetone. The combined extracts were concentrated to 10 ml. *in vacuo* at 30°; 10 ml. of petroleum ether (b.p. 30–60°) was added slowly, yielding 104 mg. of a white crystalline product (m.p. 165–166°). Recrystallization of this product from acetone–petroleum ether yielded 85 mg. of pure product VIII (m.p. 169°).

Anal. Calcd. for $C_9H_{14}O_4N_2S$: C, 43.9; H, 5.7; N, 11.4; S, 13.0. Found: C, 43.7; H, 5.8; N, 11.1; S, 12.6.

The acetone mother liquors on addition of 10 ml. of petroleum ether yielded a further 98 mg. of crystals (m.p. 166–167°). The total yield of product (202 mg.) constituted 82% of the theoretical yield.

A solution of 2 mg. of VII in 1 ml. of 0.05 *M* acetate buffer at *p*H 4.8 gave no visible color after heating at 100° with 1 ml. of 0.1% ninhydrin solution. The same initial solution gave no visible color when treated with a solution of potassium cyanide and sodium nitroprusside. An aqueous solution of VII had a *p*H of about 2.

Other Properties of Compound VI.—A solution of 10 mg. of VI in 2 ml. of water was treated with a few drops of an aqueous solution of sodium nitroprusside. Addition of 5% ammonia solution produced no purple color, even on addition of a solution of potassium cyanide. After VI (0.2 *M*) had been incubated for 2 hours at room temperature in *N* sodium hydroxide, or after boiling this solution for 30 seconds, addition of sodium nitroprusside solution produced a strong purple coloration.

A solution of 10 mg. of VI in 2 ml. of water was added to 1 ml. of 0.5 *N* hydroxylamine hydrochloride solution, then 0.1 *N* sodium hydroxide was added to *p*H 9. After incubation at room temperature for 5 minutes, acidification with dilute hydrochloric acid and addition of a few drops of 5% ferric chloride solution produced a deep red color. When the incubation was carried out at *p*H 7, no red color resulted on repeating the procedure.

NOTE ADDED IN PROOF.—After this article had been submitted for publication, we noted the abstract[24] of a paper to have been read at the April, 1960, meeting of the Federation of American Societies for Experimental Biology. In this abstract, the authors state that "NEM can react both with glycine ethyl ester and with imidazole at *p*H 7.3."

(24) R. Benesch and R. E. Benesch, *Federation Proc.*, **19**, 78 (1960).

NEW HAVEN, CONN.

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The Reaction of Amylases with Starch Granules[1,2]

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The reaction of α-amylase, β-amylase and a mixture of these with starch granules was studied. Measurements made on the residual starch and separated fractions after enzymic degradation included iodine potentiometric titration, β-amylase assay, periodate oxidation, alkali number, iodine absorption and electrophoretic studies. A method of isolating the products of enzymic digestion was developed. Evidence was presented that the enzymes in the initial stages degrade only the outer chains of amylopectin. This is best explained in terms of availability rather than enzyme mechanism. It is postulated that the surfaces of starch granules which are accessible to enzymes are the termination points of the outer chains of the amylopectin.

Introduction

Amylases react very slowly with starch granules in contrast with gelatinized starch. Nevertheless, in plants, starch granules are continually being synthesized and hydrolyzed. Reports on the nature of amylase reaction with starch granules have for the most part been confined to microscopic studies. Sandstedt[3] examined starches which had been partially digested by various enzymes. Concentric layers were clearly shown in some granules. More recently the electron microscope has been used[4,5] to reveal greater detail of partially digested starches. Observations by the

(1) Contribution No. 592, Department of Chemistry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan.

(2) Part of this paper was taken from a Masters' thesis by Yee Sik Kim, Kansas State University.

(3) R. M. Sandstedt, *Cereal Chem., Suppl.*, **32**, 17 (1955).

(4) E. S. Turner, Ph.D. Thesis, Purdue University Library, Lafayette, Indiana, 1958.

(5) Z. Nikuni, *Kagaku (Tokyo)*, **27**, 283 (1953); [*C. A.*, **51**, No. 21, 17210i (1957)]; Z. Nikuni and S. Hizukuri, *Mem. Inst. Sci. and Ind. Research, Osaka Univ.*, **14**, 173 (1957); [*C. A.*, **52**, No. 1, 766d (1958)].