$= E_3 = \ldots . E_i = E_m$. Therefore once the sites are covered by a monolayer of detergent, K^* should be equal to 1 and the BET equation for this type of binding reduces to $r_3 = nD_f/(D_c - D_f)$ which is identical in form with the empirical equation obtained above.

It is seen therefore that the values of r_2 obtained experimentally represent the sum of the detergent bound according to the manner of the second mass action step plus a small amount due to micellar binding. When the latter is neglected and a simple mass action analysis is made of the isotherm in region 2, an incorrect value for the maximum number of sites would be obtained. It is only because the CMC value for octylbenzenesulfonate greatly exceeds the D_f value at which the conversion of β-lactoglobulin to the "opened-up" form is complete that the importance of micellar type binding is recognizable.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, STATE UNIVERSITY OF IOWA]

Formation of Peptide Bonds by Aminolysis of Homocysteine Thiolactones[1]

BY REINHOLD BENESCH[2] AND RUTH E. BENESCH[2]

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It has been found that N-acylhomocysteine thiolactones can react with amines or amino acids with the formation of a peptide bond. The hydrolytic opening of the thiolactone ring can be suppressed in favor of aminolysis by using low temperatures, since the temperature coefficient of the rate of aminolysis was found to be exceptionally small. The preparation of some representative peptides in the form of their S-(phenylmercuri) derivatives is described and the possible application of the aminolytic reaction to the introduction of sulfhydryl groups into proteins is discussed.

Our interest in this field arose through a search for a method which would permit the *de novo* introduction of –SH groups into proteins under as mild conditions as possible. The only previous attempt in this direction seems to have been Schoeberl's[3] use of polythioglycolides, a rather ill-defined group of compounds. Furthermore, the data reported leave the claim that direct thiolation of protein amino groups has been accomplished by this method in serious doubt.

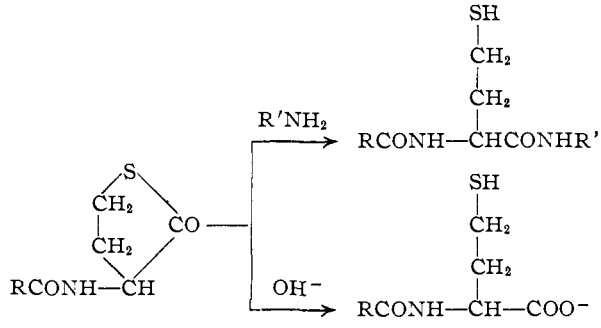
N-Substituted homocysteine thiolactones appeared to be more suitable starting materials for this purpose.[4,5]

In order to utilize these compounds for the formation of peptide bonds between homocysteine residues and the amino groups of other amino acids, peptides or proteins, several requirements should be met.

(1) The coupling should proceed under as mild conditions as possible, *i.e.*, in aqueous solution, at low temperature and as near neutrality as possible.

(2) Formation of homocysteinylhomocysteine bonds must be prevented. This was done by acylation of the amino group. The earlier experiments were performed with N-benzoylhomocysteine thiolactone.[4] This compound is, however, rather insoluble in water, particularly at lower temperatures. Therefore N-acetylhomocysteine thiolactone, which is extremely water soluble, was prepared so that the reactions could be carried out in completely aqueous solution.

(3) Hydrolytic splitting of the thiolactone ring should be avoided as far as possible, since this ring can be opened either by hydrolysis or by aminolysis



Since only the RNH_2 but not the RNH_3^+ form of an amino acid can react with the thiolactone, the solution must be sufficiently alkaline to produce an appreciable concentration of the RNH_2 species. In this *p*H range the hydrolytic opening of the ring therefore becomes a serious competitive side reaction. Kinetic studies provided a clue to this dilemma. It was found that, whereas the rate of hydrolysis has a normal temperature coefficient ($k_{t+10}/k_t \sim 2.5$), the influence of temperature on the rate of aminolysis is very much smaller ($k_{t+10}/k_t \sim 1.15$). This is illustrated by Tables I and II.

The results were obtained by following the disappearance of thiolactone at constant *p*H and with a 10-fold excess of the amine in the RNH_2 form. The half times for the disappearance of thiolactone therefore represent a comparison between the rate of hydrolysis in borate buffer and the rate of aminolysis plus hydrolysis in ammonia and glycine buffer, respectively. They clearly show that, although the rates of the two competing reactions are comparable or even identical (Table I) at elevated temperatures, the rate of hydrolysis—but not that of the aminolysis—becomes negligible at 0° (Table II). The small temperature coefficient of the rate of aminolysis may be due to the formation of an intermediate complex, the dissociation of which increases with increasing temperature (*cf.* Conant and Bartlett[6]).

(1) A preliminary account of this work was presented at the 74th Meeting of the American Society of Biological Chemists, San Francisco, California, April, 1955 (*Federation Proc.*, **14**, 487 (1955)).

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(3) A. Schoeberl, *Angew. Chem.*, **60**, 7 (1948).

(4) V. du Vigneaud, W. I. Patterson and M. Hunt, *J. Biol. Chem.*, **126**, 217 (1938).

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TABLE I

HYDROLYSIS AND AMMONOLYSIS OF N-BENZOYLHOMOCYSTEINE THIOLACTONE

Initial concentrations: thiolactone, 0.01 *M*; ethanol, 20%; NH_4OH/NH_4Cl buffer *p*H 9.4, 0.2 *M*; borate buffer *p*H 9.4, 0.2 *M*.

Temp., °C.	$t^{1/2}$ borate, min.	$t^{1/2}$ NH_3, min.
50	15	15
40	40	19
25	165	28

TABLE II

HYDROLYSIS AND AMINOLYSIS OF N-ACETYLHOMOCYSTEINE THIOLACTONE

Inital concentrations: thiolactone, 0.01 *M*; borate buffer *p*H 9.70, 0.1 *M*; glycine buffer *p*H 9.68, 0.2 *M*.

Temp., °C.	$t^{1/2}$ borate, min.	$t^{1/2}$ glycine, min.
40	36	8
25	195	12
0	∞	26

In order to study the aminolysis directly, a series of experiments was performed in which the concentrations of the thiolactone and of the amine were comparable, so that both the rate of disappearance of the amino acid and that of the thiolactone could be determined in the same solution. Some typical results are shown in Table III.

TABLE III

REACTION OF N-ACETYLHOMOCYSTEINE THIOLACTONE WITH GLYCINE

Initial concentrations: thiolactone, 0.10 *M*; borate buffer *p*H 9.6, 0.10 *M*; glycine (RNH_2), 0.02 *M*; glycine (total), 0.04 *M*.

Time, hr.	0° μmoles reacted Hydrolysis	0° μmoles reacted Aminolysis	23° μmoles reacted Hydrolysis	23° μmoles reacted Aminolysis
1.0	0.0001	0.0016	0.012	0.007
1.5	.0002	.0025	.014	.009
3.0	.0012	.0055	.020	.013
4.0	.0018	.0072	.023	.015
6.0	.0026	.0090	.029	.016
7.5	.0033	.0100	.032	.017

It can be seen that whereas under these conditions the rate of hydrolysis is considerably greater than that of the aminolysis at 23°, the reverse is true at 0°. It should be noted that in these experiments, where the concentration of amino acid was not sufficiently high to act as its own buffer, borate or carbonate buffer was used to maintain the *p*H constant. It was found, however, that both buffers—but especially the borate—inhibited the rate of aminolysis.

The information obtained from these kinetic studies was therefore used in the preparation of some representative peptides at low temperatures in good yield (*cf.* Experimental part). The peptides were isolated and analyzed in the form of their S-(phenylmercuri) derivatives. The corresponding thiols are more conveniently prepared *via* the inorganic mercury mercaptides.

Homocysteine thiolactones therefore can be used advantageously for simultaneously activating the carboxyl group and protecting the –SH group of homocysteine in forming peptide bonds with other amino acids. The reaction might be suitable for the thiolation of proteins, since it permits the introduction of an –SH-containing residue through a peptide bond at low temperatures and in completely aqueous solution.

Experimental

N-Acetyl-DL-homocysteine Thiolactone.[7]—DL-Homocysteine thiolactone hydroiodide[8] (1.225 g., 5 mmoles) and silver acetate (0.835 g., 5 mmoles) were stirred with 50 ml. of glacial acetic acid for 30 minutes at room temperature. The silver iodide was removed by centrifugation. To the supernatant solution 1 ml. of freshly distilled acetic anhydride was added and the mixture allowed to react at room temperature until the ninhydrin reaction became negative (90 minutes). The solution was evaporated to dryness *in vacuo* and the residue left over NaOH *in vacuo* overnight. The product was recrystallized from toluene; m.p. 111° (uncor.); ultraviolet spectrum: λ_{max} 238 mμ; Σ_{max} 4.4 × 10^3. It gave a negative ninhydrin reaction and the "slow nitroprusside test" typical of thiolesters.[9,10] On conversion to the hydroxamic acid it gave a purple color with ferric chloride. The R_f of the hydroxamic acid, when chromatographed on paper with water-saturated *n*-butyl alcohol as the solvent, was 0.72.

Anal. Calcd. for $C_6H_9O_2NS$: C, 45.3; H, 5.7; N, 8.8; S, 20.1. Found[11]: C, 45.4; H, 5.6; N, 8.7; S, 19.7.

S-(Phenylmercuri)-N-benzoyl-DL-homocysteine.—This compound was first prepared to test the isolation procedure *via* the S-(phenylmercuri) derivatives and also to ensure that it was possible to avoid contamination of the product with a co-precipitated excess of glycine.

N-Benzoyl-DL-homocysteine thiolactone (44 mg., 0.2 mmole), prepared according to du Vigneaud, *et al.*,[4] was heated with 4 ml. of 0.05 *N* NaOH until solution was complete and the hydroxylamine–$FeCl_3$ reaction was negative, indicating that hydrolysis of the thiolactone was complete. Sixteen ml. of water was added, the solution was acidified to congo red with 1 *N* H_2SO_4 and treated with 0.01 *N* phenylmercuric hydroxide (18 ml.) until a spot test with nitroprusside was just negative. The precipitate was collected, washed with 0.1 *N* H_2SO_4 and then with ice-cold water and dried *in vacuo* at 60°; yield 81 mg. (80%).

Anal. Calcd. for $C_{17}H_{17}O_3NSHg$: N, 2.71; Hg, 38.9; moles N/moles Hg, 1.00. Found: N, 2.52; Hg, 36.2; moles N/moles Hg, 1.00.

An identical product was obtained when a twenty-fold excess of glycine was added *after* the hydrolysis of the thiolactone and the subsequent isolation procedure followed as described above.

S-(Phenylmercuri)-N-benzoyl-DL-homocysteinylglycine.—N-Benzoyl-DL-homocysteine thiolactone (44 mg., 0.2 mmole) was dissolved in 8 ml. of ethanol and 12 ml. of 0.33 *M* glycine buffer *p*H 9.6 was added. The solution was freed of oxygen by passage of nitrogen and left at 0° for 12 hours, at which time the hydroxylamine–$FeCl_3$ test was negligible. The clear solution was acidified to congo red with 1 *N* H_2SO_4 and concentrated *in vacuo* below 50° to about 1/4 of the original volume. Precipitation with phenylmercuric hydroxide and isolation of the compound was carried out as described above; yield 57 mg. (59%). A second crop can be obtained by concentrating the filtrate from the first crop. The absence of free glycine from the final product was ascertained by a negative ninhydrin test and the absence of a glycine spot on paper chromatography.

Anal. Calcd. for $C_{19}H_{20}O_4N_2SHg$: N, 4.89; Hg, 35.0; moles N/moles Hg, 2.00. Found: N, 4.50; Hg, 32.4; moles N/moles Hg, 1.99.

S-(Phenylmercuri)-N-benzoyl-DL-homocysteinylglycylglycine.—N-Benzoyl-DL-homocysteine thiolactone (44 mg., 0.2 mmole) was dissolved in 4 ml. of ethanol and 16 ml. of 0.25 *M* glycylglycine buffer *p*H 8.3 was added. The mix-

(7) This compound is now available from Schwarz Laboratories, Mount Vernon, New York.

(8) H. D. Baernstein, *J. Biol. Chem.* **106**, 451 (1934).

(9) F. Lynen and E. Reichert, *Angew. Chem.*, **63**, 47 (1951).

(10) F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1950).

(11) Analyses were performed by Microtechnical Laboratories, Skokie, Ill.

ture was freed of oxygen and left at 30°[12] until the hydroxylamine–$FeCl_3$ test was negative (20 hours). The compound was again isolated by the procedures described above; yield 66 mg. (65%).

Anal. Calcd. for $C_{21}H_{23}O_5N_3SHg$: N, 6.67; Hg, 31.8; moles N/moles Hg, 3.00. Found: N, 6.76; Hg, 30.7; moles N/moles Hg, 3.15.

Analyses.—Nitrogen was determined by Kjeldahl. The mercury analyses were performed on 8–10 mg. samples as described previously.[13]

Kinetic Experiments.—In the series of experiments in which the amine was in excess (Tables I and II), the concentration of thiolactone was 0.01 *M* and the total concentration of amine was 0.2 *M* of which half was in the RNH_2 form.[14] The experiments with N-benzoylhomocysteine thiolactone were carried out in 20% ethanol and those with N-acetylhomocysteine thiolactone in completely aqueous solution. The disappearance of thiolactone was followed either from the absorption at 238 mμ[15,16] in the experiments in which N-acetylhomocysteine thiolactone was used, or with the hydroxylamine–$FeCl_3$ reaction in the case of N-benzoylhomocysteine thiolactone, where the high absorption of the benzamido group makes the ultraviolet method less suitable.

For the measurement of the absorption at 238 mμ 0.2-ml. samples of the reaction mixtures were withdrawn at different time intervals and immediately added to 1 ml. of 0.5 *M* acetate buffer *p*H 4. After dilution to 10 ml., the optical density was determined in a Beckman DU spectrophotometer. The acid buffer served the double purpose of stopping the reaction and preventing the interfering absorption of RS^-.[16]

The reaction with hydroxylamine was based on the method of Lippman and Tuttle.[17] An aliquot of the reaction mixture (0.5 ml.) was allowed to react at room temperature for 20 minutes with 2.5 ml. of a $NH_2OH/ClNH_3OH$ buffer (1.0 *M*, *p*H 5.5). The time interval was sufficient for complete conversion to the hydroxamic acid. The solution was treated with 1 ml. of 25% HCl and 1 ml. of 5% $FeCl_3 \cdot 6H_2O$ in 0.1 *N* HCl and the optical density at 515 mμ was measured after 10 minutes.

In the experiments in which thiolactone and amino acid disappearance were followed simultaneously (Table III), the former was again determined by its ultraviolet absorption. The latter was assayed with the modified ninhydrin reagent of Moore and Stein.[18] This was the only ninhydrin method, of several investigated, which gave satisfactory results in the presence of a free–SH group. Aliquots of the reaction mixture were mixed with an excess of acetate buffer *p*H 5.5 and appropriate dilutions were made for ultraviolet spectrophotometric and ninhydrin assays. In this way the rate of aminolysis plus hydrolysis could be obtained from the ultraviolet data and that of the aminolysis alone from the ninhydrin determination. Thus the rates of hydrolysis and aminolysis could be calculated separately (Table III).

(12) This higher temperature was chosen for the preparation of this compound because the alcohol concentration necessary for the dissolution of the N-benzoylhomocysteine thiolactone at 0° is too high to dissolve appreciable amounts of glycylglycine. It will be noted, however, that, in view of the lower *p*K of glycylglycine, the reaction could be carried out at *p*H 8.3, so that even at 30° the hydrolysis does not constitute a serious side reaction.

(13) R. Benesch and R. E. Benesch, *Arch. Biochem. Biophys.*, **38**, 425 (1952).

(14) Th *p*H of such a mixture varies, of course, quite considerably with temperature (ΔH-NH_2 ~10 kcal.). In the studies at different temperatures the RNH_2 concentration rather than the *p*H was kept constant.

(15) B. Sjoeberg, *Z. physik. Chem.*, **52B**, 209 (1942).

(16) L. H. Noda, S. A. Kuby and H. A. Lardy, THIS JOURNAL, **75**, 913 (1953).

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(18) S. Moore and W. H. Stein, *ibid.*, **211**, 907 (1954).

IOWA CITY, IOWA

[CONTRIBUTION FROM THE LANKENAU HOSPITAL RESEARCH INSTITUTE AND THE INSTITUTE FOR CANCER RESEARCH]

A Study of Leucine Biosynthesis in *Torulopsis utilis*[1]

BY MURRAY STRASSMAN,[2] LILLIAN A. LOCKE, ALICE J. THOMAS AND SIDNEY WEINHOUSE

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Leucines isolated from the proteins of yeast cells grown on glucose as the principal carbon source, together with tracer amounts of variously C^{14}-labeled acetic and lactic acids, were found to be highly active. The distributions of activity among the individual carbon atoms of these leucines suggested that carbons one and two of the leucine skeleton arise from the respective acetate carboxyl and methyl carbons, and the other four carbons originate from the isobutyl portion of valine. A mechanism for leucine biosynthesis is proposed, involving a condensation of α-ketoisovaleric acid with acetyl CoA to yield, ultimately, α-ketoisocaproic acid by a series of reactions analogous to those of the citric acid cycle.

The similarity in the structures of the branched chain amino acids, valine, leucine and isoleucine, suggests that these substances may be products of closely related biosynthetic pathways. In previous studies, evidence from isotope tracer experiments was presented to indicate that valine and isoleucine may be synthesized *via* a similar series of reactions. In the previous study of valine biosynthesis, growth of *Torulopsis utilis* on glucose as essentially the sole carbon source, together with tracer quantities of lactic acid-1-, 2-, or 3-C^{14}, yielded highly labeled valines with the following distribution of lactate carbons; the carboxyl carbon was derived from the lactate carboxyl, carbons 2 and 3 were derived equally from the lactate α-carbon and the methyl carbons were derived from the lactate methyl carbon. It was found and reported in a preliminary communication[3] that the leucines isolated from the same experiments have the same distribution of lactate carbons 2 and 3 in carbons 3 to 5,5′ of leucine as was previously observed in carbons 2 to 4,4′ of valine. Moreover, the carboxyl and methyl carbons of acetic acid were incorporated readily and equally in carbons 1 and 2, respectively, of leucine. The present report represents a more detailed account of this study.

Abelson[4,5] has recently found that the addition of acetic or pyruvic acid, valine or its keto analog to the growth medium of *Escherichia coli*, *T. utilis* and *Neurospora crassa* growing on uniformly C^{14}-labeled glucose markedly lowered the incorporation of C^{14} in leucine. He suggested a metabolic se-

(1) This work was supported by the United States Atomic Energy Commission, Contract No. At(30-1)777 and was aided in part by grants from the National Institutes of Health and the American Cancer Society.

(2) Much of this study was conducted during the tenure of a Post Doctoral Fellowship from the National Institutes of Health.

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(5) P. H. Abelson and H. J. Vogel, *ibid.*, **213**, 355 (1955).