with dilute formic acid on the filter. The dried sample melted at 204–208°.[3]

Anal.[6] $C_{26}H_{38}O_7$: C, 67.51; H, 8.28. Found: C, 67.14; H, 8.18.

Summary

The preparation of ethyl 3,12-dihydroxy-7-ketocholanate is described and subsequent hydrolysis of the ester yielded 3,12-dihydroxy-7-ketocholanic acid.

The various melting points described for the 3,12-dihydroxy-7-ketocholanic acid are discussed.

The hitherto unknown 3,7-diketo-12-hydroxycholanic acid is described.

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Action of Aromatic Isocyanates on Proteins

BY HEINZ FRAENKEL-CONRAT, MITZI COOPER AND HAROLD S. OLCOTT

Aromatic isocyanates in anhydrous media are widely used for the characterization of alcohols and amines. However, since the amino group of amino acids reacts smoothly with isocyanates only in aqueous alkaline solution,[2] proteins were usually treated with isocyanates at *p*H 8–9. Hopkins and Wormall[3] showed that under mild conditions the reaction involves the amino groups almost exclusively, while others[4] have since observed that phenolic and thiol groups also may react.

It has been found in this Laboratory that in the absence of water and at elevated temperature the treatment of proteins with phenyl isocyanate causes the introduction of considerably more of the reagent than can be accounted for by the number of amino, thiol, and phenolic groups known to be present. Determination of the specific groups responsible for the unexpected extent of the reaction was the objective of this investigation.[5]

In general, the reaction was accomplished by heating a suspension of the dry protein in phenyl isocyanate and pyridine for twenty-four hours at 70°.[6] Four criteria were used to measure the extent of the interaction: (1) The weights of the protein samples were increased through addition of the isocyanate. The final weight gains after washing and extracting varied to a certain extent with the solubility characteristics of the original proteins, but, for any one protein, the extent of interaction was expressed clearly by the amount of reaction product isolated. (2) Chlorine analyses after treatment with chlorophenyl isocyanates furnished an alternate measure of the over-all extent of interaction, independent of other properties of the protein. On this basis hoof powder bound *o*- and *p*-chlorophenyl isocyanates to the extent of 39 and 45% of its weight, respectively. Such analyses were particularly useful in determining the small amount of combination of isocyanates with substances containing few reactive groups. (3) The decrease in hydrophilic property was found to be indicative of the extent of reaction.[5] (4) Protein-group analyses permitted a measure of the degree of substitution of specific polar groups. All types of acid and basic, primary amide, and, indirectly, peptide nitrogen groups were studied.

Reaction with Basic and Acid Groups.—The study of the nature of the reaction was facilitated by newly developed methods for the determination of total acid and basic groups applicable to insoluble proteins.[7] A comparison of the number of basic groups of treated and untreated proteins revealed the fact that these had been almost completely abolished by the treatment (Table I). Thus not only the primary amino groups of lysine, but also the guanidyl and imidazole residues of arginine and histidine, appeared to react with isocyanates under the conditions used. Since insulin is particularly rich in histidine, the loss of most of its basic groups was confirmatory evidence of the reactivity of the imidazole group.

In addition the number of acid groups of proteins was greatly decreased. The magnitude of this effect indicated that the carboxyl, as well as the thiol and phenolic groups, had reacted. The action of aromatic isocyanates on simple carboxylic acids has been repeatedly studied.[8] The reaction follows two paths, one resulting in the formation of acyl anilides and carbon dioxide and the other in the formation of anhydrides, N,N'-diphenyl ureas and carbon dioxide. Experiments carried out on amino acid derivatives in general confirmed these findings.

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Paal, *Ber.*, **27**, 974 (1894); **38**, 2359 (1905).

(3) Hopkins and Wormall, *Biochem. J.*, **27**, 740 (1933); **28**, 2125 (1934).

(4) (a) Miller and Stanley, *J. Biol. Chem.*, **141**, 905 (1941); (b) Fraenkel-Conrat, *ibid.*, **152**, 385 (1944).

(5) The products of the anhydrous reaction of phenyl isocyanate and proteins were water-resistant to the extent that they appeared to have promise in the field of plastics. Data on this phase of the subject will be presented elsewhere.

(6) The use of pyridine was suggested by the work of Hearon, Hiatt and Fordyce (THIS JOURNAL, **65**, 829 (1944)) who studied the anhydrous reaction of phenyl isocyanate with cellulose. Control experiments, in connection with the work here reported, indicated that the polar groups of proteins were not affected by pyridine alone.

(7) Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, **154**, 239 (1944).

(8) The literature has been reviewed by Naegeli and Tyabji, *Helv. Chim. Acta*, **17**, 931 (1934).

TABLE I

EFFECT OF PHENYL ISOCYANATE TREATMENT[a] ON THE ACID AND BASIC GROUPS OF PROTEINS

Protein	Acid groups[b] Untreated	Acid groups[b] Treated	Basic groups[b] Untreated	Basic groups[b] Treated
Egg albumin	13.5	0.4	8.8	0.0
Gliadin[c]	4.3	2.7	4.7	1.1
Gluten[c]	6.7	2.2	4.3	0.5
Cattle hoof keratin[d]	10.4	1.6	8.8	1.0
Silk fibroin[e]	8.4	1.3	1.3	0.0
Casein[c]	15.6	0.7	7.4	0.7
Insulin	17.5	2.3	9.4	0.0

[a] The proteins were treated with phenyl isocyanate and pyridine in the ratio 1:1:2.5 for two days at 70°. Yields ranged from 115% for gluten to 136% for egg albumin. [b] Per g. protein $\times 10^4$. The percentage of error in these determinations was considerably greater with protein samples containing very small numbers of residual polar groups than with the unreacted proteins. [c] Lyophilized from dilute acetic acid solution. [d] When the cattle-hoof powder (100 mesh) was treated with phenyl isocyanate at 70° in the absence of pyridine, the acid and basic groups remaining after two days were 4.1 and 1.1, and after 7 days, 2.9 and 1.3, respectively. [e] *o*-Chlorophenyl isocyanate was used in this experiment. The chlorine content indicated that 15.6 g. equivalents of the reagent had been introduced into 10^4 g. of protein. The yield was 114%. Another batch of silk fibroin, treated with phenyl isocyanate, gained only 4% in weight; the acid groups of that derivative were 4.3, the basic groups, 0.0.

In order to determine whether the carboxyl groups of proteins reacted similarly with phenyl isocyanate, the following experiment was undertaken. A naturally occurring polypeptide, the polar groups of which are almost exclusively free carboxyl groups,[9] was treated with *o*-chlorophenyl isocyanate. The di-*o*-chlorophenyl urea, obtained as a by-product, and the chlorine content of the resultant product were quantitatively determined. If the formation of acyl anilides were the dominant reaction, only small amounts of substituted diphenyl urea would be formed, and the chlorine content of the product would approach that equivalent to the loss in acid groups. On the other hand, if anhydride formation were the dominant reaction, substituted diphenyl urea would be formed in appreciable amounts, and the chlorine content of the peptide derivative would be low. The results, given in detail below, suggest that both reactions occurred with protein carboxyl groups under the conditions used.

The ability of the phenolic groups to react with isocyanates was clearly demonstrated in the case of silk fibroin, the acid groups of which are predominantly the hydroxyl groups of tyrosine. This protein also supplied indirect evidence for the reactivity of the aliphatic hydroxyl groups. For it was possible to introduce into a fibroin preparation an amount of *o*-chlorophenyl isocyanate which was about double that equivalent to its acid and basic groups but less than equivalent to the sum of these and its aliphatic hydroxyl groups.

(9) A polymer of glutamic acid, Bovarnick, *J. Biol. Chem.*, **145**, 451 (1942).

Reaction with Primary Amide Groups.—While the total number of acid, basic and hydroxyl groups may be sufficient to account for the extent of interaction of phenyl isocyanate with proteins rich in these groups, such as egg albumin, it is insufficient with proteins containing few of these types of polar groups, such as gliadin and zein. The latter proteins, however, contain considerable numbers of primary amide groups. The reaction of these groups with phenyl isocyanate therefore appeared probable.

The reaction of isocyanates with simple amides has not been extensively studied since it was first described by Kuehn,[10] who considered it an addition reaction similar to that of phenyl isocyanate with amines. That it occurs under our experimental conditions was indicated by the ready synthesis of several N,N'-acyl phenyl ureas from acyl amides and phenyl isocyanate in good yields.

For the purpose of demonstrating that protein amide groups are similarly affected, advantage was taken of the difference in reactivity of free and substituted amides toward nitrous acid. Plimmer[11] has shown that primary acid amides yield their nitrogen quantitatively when treated for twenty-four hours at room temperature with nitrous acid in the presence of mineral acid. Amines and guanidyl derivatives react similarly. When this technique was applied to proteins, the nitrogen evolved was approximately equivalent in amount to their total contents of amide, amino, and guanidyl nitrogen (Table II). In contrast to this, phenyl isocyanate-treated proteins under the same conditions yielded little nitrogen. These findings, particularly with proteins rich in amide and poor in basic groups,

TABLE II

NITROGEN LIBERATED FROM VARIOUS PROTEINS AND THEIR PHENYL ISOCYANATE DERIVATIVES BY TREATMENT WITH NITROUS ACID IN 2 *N* HYDROCHLORIC ACID DURING TWENTY-FOUR HOURS AT 21–24°

Protein	Calculated for original protein,[a] % Amide N	Calculated for original protein,[a] % Amide + amino + guanidyl N	Found, % Original protein	Found, % Phenyl isocyanate derivative
Gliadin	4.3	5.1	5.4 (4)[b]	1.6[c] (2)
Egg albumin	1.0	2.9	3.0 (2)	0.8 (2)
Casein	1.3	2.8	3.3 (2)	1.9 (2)
Zein	3.0	3.4	3.5 (2)	0.8 (2)
Gluten	3.0	4.2	4.1 (9)	0.8 (5)

[a] The data for amino and amide nitrogen were determined; those for guanidyl were taken from Cohn and Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, pp. 354–360. [b] Figures in parentheses represent numbers of analyses which, in general, did not deviate by more than 10% from the average. [c] After phenyl isocyanate treatment in aqueous solution, the nitrogen liberated was 3.8% (2).

(10) Kuehn, *Ber.*, **17**, 2881 (1884); Kuehn and Henschel, *ibid.*, **21**, 504 (1888).

(11) Plimmer, *J. Chem. Soc.*, **127**, 2651 (1925).

TABLE III

EFFECT OF PHENYL ISOCYANATE ON WATER-SOLUBLE EGG WHITE PROTEINS

Experiment	Pretreatment	Treatment[a]	Yield, %	Groups per 10^4 g. of protein[a] Acid	Basic	Amino
		In Anhydrous Media				
1	Lyophilized	Pyridine, 70°, 48 hours	84	11.8	6.0	1.4
2	Adjusted to *p*H 4.5, lyophilized	Pyridine, 70°, 48 hours	99	4.7	2.4	0.9
3	Adjusted to *p*H 4.5, dialyzed, lyophilized	Pyridine, 70°, 48 hours	137	2.0	0.0	0.1
4	Adjusted to *p*H 4.5, dialyzed, lyophilized	Pyridine, 70°, 24 hours	134	0.9	0.4	0.2
5	Adjusted to *p*H 4.5, dialyzed, lyophilized	No pyridine, 70°, 96 hours[b]	110	6.0	4.6	1.6
6	Adjusted to *p*H 4.5, dialyzed, lyophilized	No pyridine, 70°, 24 hours	107	7.8	5.0	3.2
7	Adjusted to *p*H 4.5, dial., then to *p*H 3.1, lyoph.	Pyridine, 70°, 48 hours	136	0.3	0.7	0.3
8	Adjusted to *p*H 4.5, dial., then to *p*H 7.2, lyoph.	Pyridine, 70°, 48 hours	92	2.2	2.1	0.9
9	Adjusted to *p*H 4.5, dial., then NaCl added, lyoph.	Pyridine, 70°, 48 hours	139	0.5	0.4	0.0
10	Electrodialyzed, lyophilized	Pyridine, 70°, 48 hours	125	2.0	0.9	0.1
11	Electrodialyzed, lyophilized	No pyridine, 70°, 48 hours[b]	122	1.5	0.3	0.3
12	Electrodialyzed, lyophilized	Pyridine, 23°, 20 hours	102	6.4	1.2	0.2
13	Electrodialyzed, lyophilized	No pyridine, 23°, 20 hours	70	10.3	4.8	1.4
14	Electrodialyzed, lyophilized	None (control)	...	11.6	8.5	4.5
		In Aqueous Media[c]				
15	Electrodialyzed, lyophilized	No buffer added, *p*H 4.8, 23°, 20 hours	80[d]	10.0	4.6	1.4
16	Electrodialyzed, lyophilized	Acetate buffer, *p*H 3.8, 23°, 20 hours	20[d]	9.7	5.0	1.2
17	Electrodialyzed, lyophilized	Phosphate buffer, *p*H 8.0, 23°, 20 hours	88[d]	11.4	3.6	0.3
18	Electrodialyzed, lyophilized	10 per cent. pyridine, 23°, 20 hours	42[d]	10.6	6.8	2.3

[a] See Table I, footnotes *a* and *b*. [b] Treatment for at least two days, without pyridine, led to variable results for different batches. [c] Reagent to protein ratio = 2:1. [d] Amounts of protein rendered water insoluble by the treatment

appear to furnish proof that most of the amide groups have reacted with phenyl isocyanate.[12]

Reaction with Peptide Bonds.—When the amide nitrogen of proteins was found to react with phenyl isocyanate, it appeared possible that the secondary amide groups of the peptide chain might be similarly involved. That this reaction did not occur to any considerable extent was indicated by the finding that the amounts of phenyl isocyanate introduced into various proteins corresponded approximately to their content of reactive side chains. To further substantiate this finding model peptides, which differed from proteins in that they contained no groups known to react with phenyl isocyanate, were treated with this reagent or with *o*-chlorophenyl isocyanate. The substances chosen were (1) polymers of glycine, (2) Nylon molding powder (a polyamide of adipic acid and hexamethylenediamine) and (3) diketopiperazines. The various types of chain polymers appeared to react only to a limited extent, as indicated by their unchanged nitrogen contents, or by the small amounts of chlorine introduced. On the other hand, the cyclic anhydrides of glycine and alanine bound one molecule of phenyl isocyanate for each peptide bond.[13] These findings support the belief that typical peptide bonds of proteins show little tendency to react with aromatic isocyanates.

Reaction with Proteins in Aqueous Solution.—To permit comparisons between the phenyl isocyanate derivatives of proteins made under anhydrous conditions with those prepared in water solution,[3,4] several preparations obtained by the latter technique were also studied analytically. The data listed in Table III (expts. 15–18) were obtained on the fraction of the egg white proteins that was rendered insoluble through the treatment. They indicate that there is little reaction of the acid groups in an acid medium, and none in alkaline solution. The reactivity of the primary amino groups, on the other hand, was favored by the alkaline medium. Other basic groups appeared not to react appreciably in water. Since pyridine causes the immediate reaction of phenyl isocyanate with water, its presence in experiments in aqueous solution was of no advantage.

Determinations of the thiol and phenolic groups were restricted to the soluble fraction of

(12) The classical method for the determination of protein amide nitrogen through partial hydrolysis could not be applied successfully to the protein derivatives under investigation, probably due to the lability of the acyl urea linkages. Thus N,N'-acetyl phenyl urea was found to yield 89% of its nitrogen as ammonia after treatment with 6% sulfuric acid for eighty minutes at 120°, conditions which we have found suitable for the determination of amide nitrogen in proteins.

(13) Richardson and Welch (THIS JOURNAL, 51, 3074 (1929)) prepared a derivative of diketopiperazine with 2 molecules of α-naphthyl isocyanate. They regarded this reaction as proof of an enolic structure of the ring, without taking into account the possibility that the isocyanate might react with the imide group rather than the enol-hydroxyl group. While the true mode of linkage is unknown, it appeared of no immediate consequence for the problem under investigation whether isocyanate combines with the imide or enol form of peptides, but rather whether it reacts at all.

the treated protein, since they were done colorimetrically. Egg albumin was therefore treated with small amounts of phenyl isocyanate in order to cause precipitation of less than half of the protein. The thiol groups of the soluble fraction were decreased both at *p*H 3.3 and *p*H 8.0,[14] but the phenolic groups appeared unreactive toward phenyl isocyanate, in contrast to those of tobacco mosaic virus.[4a] The amino groups of the soluble fraction were reduced to a lesser extent than were those of the insoluble fraction. At *p*H 3.8, no loss of free amino nitrogen was observed; at *p*H 8.0, the amino groups were decreased by 75%.

Treatment of gliadin in 0.1 *N* acetic acid solution with phenyl isocyanate led to a less extensive reaction of the amide groups than occurs in anhydrous medium (Table II, *c*).[15]

Experimental

Materials.—Crystalline egg albumin, prepared according to Kekwick and Cannan,[16] was supplied by Dr. F. E. Lindquist. Egg white was obtained from fresh eggs. Gliadin was prepared from wheat gluten by a method of selective precipitation which will be described elsewhere. Cattle hoof powder was prepared by wet grinding.[17] Zein, gluten and casein were commercial products. Silk fibroin was kindly supplied by Dr. M. Bergmann; crystalline zinc insulin by Eli Lilly and Company.

The preparation of polyglycine ester is described below. Nylon powder (finer than 80 mesh) was obtained by prolonged grinding of the commercial molding powder in a ball mill. Polyglutamic acid was prepared in coöperation with J. C. Lewis from a bacterial culture medium as described by Bovarnick.[9]

Technique of Treatment with Phenyl Isocyanate.—The protein sample was dried for two to four hours at 100°, then overnight at room temperature over phosphorus pentoxide *in vacuo*. An approximately equal amount of phenyl isocyanate (1 ml. per g. of protein) was added, together with two-and-a-half times as much pyridine, which had been dried by storage in contact with barium oxide. The mixture was allowed to react for twenty-four hours at 70°. Dry toluene was then added; the protein was separated by filtration or centrifugation, washed once with toluene and once with ethanol, and subsequently extracted for one day with ethanol[18] in a Soxhlet extractor. The product was finally dried with ether and in an oven at 50°. The yields varied from 100 to 140% depending upon the amount of interaction and, to a lesser extent, upon the solubility of the original protein in the reagents used.

Certain variations in technique were found permissible. Removal of water could be achieved in different ways, but it was important that both protein and reagents be dry, since pyridine catalyzed the reaction of phenyl isocyanate with water. The amount of phenyl isocyanate could not be decreased to less than half if maximal reaction was intended; an increase was of no advantage. The reaction could take place at 100° instead of at 70°; it then approached completion within six to eight hours.

The removal of excess phenyl isocyanate could be accomplished most conveniently by washing the reaction product with an inert solvent, such as toluene or benzene. However, distillation or reaction with water or ethanol was also effective. The object of the subsequent extraction with ethanol or ether was the removal of the N,N'-diphenyl urea which in small amounts is a by-product and which forms in considerable amounts from the excess phenyl isocyanate if water is used in terminating the reaction.

Although phenyl and *o*-chlorophenyl isocyanates were used exclusively in the experiments reported in this paper, other cyclic isocyanates also have been prepared and reacted with proteins. These included *o*-biphenyl, *o*-bicyclohexyl,[19] β-anthryl,[20] *p*-cetyl phenyl[21] and several commercial isocyanates. Greater weight gains were obtained with *o*-biphenyl and *p*-cetyl phenyl isocyanates in approximate proportion to their greater molecular weights. Some, in particular *o*-bicyclohexyl isocyanate, reacted poorly. With others, difficulties were encountered in the separation of very insoluble by-products from the protein derivatives.

The physical state and method of preparation of a protein were found to be significant although not always clearly understood factors in determining the rate and extent of its reaction with phenyl isocyanate. For example, native gluten, gliadin and casein were considerably more reactive after they had been lyophilized from dilute acetic acid solutions than when used without such pretreatment. Denatured gluten was more reactive than native gluten. It appeared that each protein required separate study to determine the conditions favoring maximal reaction.

Thus, although crystalline egg albumin reacted readily with phenyl isocyanate, lyophilized egg white reacted only to a very limited extent (Table III). Electrodialysis, or simple dialysis subsequent to the adjustment of the *p*H of the solution to that reached through electrodialysis (approximately *p*H 5), rendered the egg white proteins reactive. Re-addition of the various substances which had been removed from egg whites by these procedures indicated that the presence of alkali could well account for the non-reactivity of untreated egg white (Table III, Expt. 8).

For the experiments in which egg albumin in aqueous solution was treated with phenyl isocyanate, the technique used was that described previously[4b]; the solutions were adjusted to the proper *p*H with acetate and phosphate buffers.

Determination of Total Amide, Guanidyl and Amino Nitrogen of Proteins and Limitations of the Method.—Following the directions of Plimmer,[11] the materials (100 to 300 mg. in the case of proteins) were treated with sodium nitrite in 2 *N* hydrochloric acid in a macro-volumetric Van Slyke apparatus. The reaction was permitted to proceed for twenty-four hours with occasional shaking. Correlation of the amounts of nitrogen evolved with the total primary amide, amino, and the guanidyl nitrogen contents of several proteins is indicated in Table II. In agreement with Plimmer, simple amides, including asparagine and glutamine derivatives, were quantitatively determinable. However, the results of a number of model experiments indicated that some compounds yielded nitrogen that was not derived from amide, amino, or guanidyl groups. This was the case with glutathione, tyrosine, tryptophan, diketopiperazines and some N,N'-disubstituted ureas.[22]

The secondary amide groups of diketopiperazines were found to liberate nitrogen in amounts that appeared to be correlated with the lability of the compounds toward acid

(14) Previous experiments had shown that thiol groups reacted with acylating agents, including phenyl isocyanate, at *p*H 5-6.[4b]

(15) The reaction of protein amide groups with phenyl isocyanate in aqueous solution has not previously been demonstrated. Jensen and Evans (*J. Biol. Chem.*, **108**, 1 (1935)) showed that ammonia was not liberated as readily from phenyl isocyanate-treated as from untreated insulin. This might possibly have been due to the combination of labile amide groups with phenyl isocyanate.

(16) Kekwick and Cannan, *Biochem. J.*, **30**, 227 (1936).

(17) Olcott, *Proc. Soc. Exptl. Biol. Med.*, **54**, 219 (1943).

(18) Ether was used with alcohol-soluble proteins, such as gliadin and zein.

(19) Fraenkel-Conrat and Olcott, THIS JOURNAL, **66**, 845 (1944).

(20) Fieser and Creech, *ibid.*, **61**, 3502 (1939).

(21) Coffey and Haddock, U. S. Patent No. 2,118,493 (1938); Greenhalgh and Piggett, U. S. Patent 2,311,046 (1943).

(22) Glutathione was shown by Hopkins (*J. Biol. Chem.*, **84**, 271 (1929)) to give high amino nitrogen values. The same was found to be the case with tyrosine in the presence of light (Fraenkel-Conrat, *J. Biol. Chem.*, **148**, 453 (1943)). The effect of light was confirmed also in the present study, since tyrosine yielded almost the theoretical amino nitrogen when treated in the dark, but about 150% when treated in daylight by Plimmer's technique.

hydrolysis. Glycine anhydride, the least stable, yielded both nitrogen atoms; the anhydrides of other amino acids yielded approximately one. In contrast to the cyclic peptides, the polyglycine ester did not yield its peptide nitrogen under these conditions.

Of the N,N'-substituted ureas, which were tested as models for similar groups introduced into proteins through combination with phenyl isocyanate, diphenyl urea yielded the highest spurious amide nitrogen value (60% of the total nitrogen). Acetyl phenyl urea yielded 30% of its nitrogen, and enanthyl phenyl urea yielded 17%, while stearyl phenyl urea was stable to nitrous acid in 2 *N* hydrochloric acid.

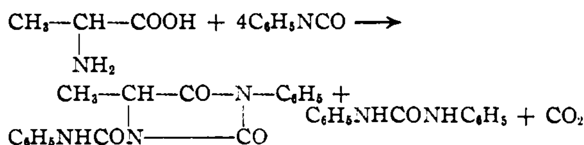
The observations mentioned have led to the following conclusions: Under the conditions described, proteins treated with sodium nitrite and hydrochloric acid may be expected to yield an amount of nitrogen equivalent to their entire primary amino and amide nitrogen, plus three-fourths of their arginine nitrogen, plus a smaller and less well-defined fraction of their tyrosine and tryptophan contents and, possibly, some of their "most labile" peptide bonds. In the case of phenyl isocyanate-treated proteins, some of the substituting carbanilo groups may also yield "amide" nitrogen. However, as indicated by the data in Table II, reactions of nitrous acid other than with primary amide and amino and guanidyl groups probably play only a small role in the analysis of proteins.

Preparative

Reaction of Phenyl Isocyanate with Alanine: 1-Carbanilo-3-phenyl-5-methylhydantoin.—To 2.0 g. of *dl*-alanine, dried at 100°, was added 8 ml. of phenyl isocyanate and 14 ml. of dry pyridine. Reaction was indicated by development of heat and liberation of a gas. The mixture was heated to 70° for two hours, then extracted with warm carbon tetrachloride. Of the undissolved residue (3.7 g.), 85% was N,N'-diphenyl urea. From the carbon tetrachloride extract, 2.45 g. of the crude hydantoin crystallized upon cooling (m. p. 150°). One recrystallization from ethanol yielded 2.25 g. of the pure substance; m. p. 155–156° (uncor.). The material could also be recrystallized from mixtures of ethyl acetate or methyl alcohol and petroleum ether.

Anal. Calcd. for $C_{17}H_{15}N_3O_3$: C, 66.0; H, 4.85; N, 13.6; mol. wt., 309. Found: C, 66.0; H, 4.79; N, 13.5; mol. wt. (Rast), 256, 260.

The equation for the reaction is probably



The amount of phenyl isocyanate needed for quantitative conversion according to this equation would be about 11 ml. (5.35 times the weight of the alanine); the maximal yield of the hydantoin would then be 6.9 g.

The tendency of phenylhydantoic acids to become cyclized with loss of water is well known, and the reactivity of the —NH— group in the ring has recently been pointed out.[23]

Reaction of Phenyl Isocyanate with Acyl Amides: N,N'-Acylphenyl Ureas.—A 5.0-g. sample of commercial heptamide and a similar sample of stearamide, after being dried by vacuum evaporation of their solutions in toluene were treated with 5-ml. quantities of phenyl isocyanate at 100° for forty-eight hours. The homogeneous reaction mixtures solidified upon cooling. Recrystallization from methanol and toluene, respectively, gave enanthyl phenyl urea and stearyl phenyl urea in 70 to 80% yields. The presence of pyridine did not appear to benefit the reaction of simple amides with phenyl isocyanate.

(23) Szabo and Karabinos, THIS JOURNAL, **66**, 560 (1944).

Reaction of Phenyl Isocyanate with Diketopiperazines:[24] (a) **Dicarbanilo-2,5-piperazinedione.**—A closed vessel containing 0.4 g. of glycine anhydride and 2 ml. of phenyl isocyanate was placed in an oven at 100° for two days. Excess reagent was removed by vacuum distillation. The white residue (1.14 g.) was recrystallized from toluene. The pure condensation product weighed 0.82 g.; m. p. 315° (uncor.). It was insoluble in ether.

Anal. Calcd. for $C_{18}H_{16}O_4N_4$: N, 15.9; amino N after hydrolysis, 8.0. Found: N, 15.8; amino N (Van Slyke) after hydrolysis, 8.3.[25]

When the reaction was carried out at 70° in the presence of pyridine, it was incomplete after one day, as indicated by a weight increase of only 17% over that of the anhydride used and by a nitrogen content of 21.7%.

(b) **3,6-Dimethyl-dicarbanilo-2,5-piperazinedione.**—A 0.5-g. sample of alanine anhydride was treated with 0.7 ml. of phenyl isocyanate and 1.4 ml. of dry pyridine at 70° for one day. The mixture was extracted with cold dry toluene, then in a Soxhlet extractor with ether. The ether was evaporated and the residue was recrystallized from much ethanol. The pure condensation product weighed 0.24 g.; m. p. 177–178° (uncor.).

Anal. Calcd. for $C_{20}H_{20}O_4N_4$: N, 14.7; amino N after hydrolysis, 7.4. Found: N, 14.6; amino N (Van Slyke) after hydrolysis, 7.5.[26]

The same compound was obtained also by the method used with glycine anhydride (110 mg. from 73 mg. of alanine anhydride), except that the crude condensation product was recrystallized directly from ethanol.

Reaction of *o*-Chlorophenyl Isocyanate with Polyglutamic Acid.—To 0.75 g. of dry polyglutamic acid[9] was added 2.5 ml. of *o*-chlorophenyl isocyanate and 5.5 ml. of dry redistilled pyridine in a gas wash bottle. In a second experiment the amounts were 0.5 g. and 2 and 5 ml., respectively. The containers were closed and held at 70° for one and two days, respectively. The amount of carbon dioxide evolved was determined semi-quantitatively and the reaction mixtures were then worked up as described for proteins; the substituted urea was found to be completely extracted by ether in two days. N,N'-Di-*o*-chlorophenyl urea was isolated quantitatively from the ether by alternate filtration and concentration. The extracted reaction products (0.72 g. and 0.48 g.) were analyzed for chlorine and for acid groups. Found in the two experiments, respectively: gram equivalents of anhydrides formed (*i. e.*, moles of di-*o*-chlorophenyl urea formed), approximately 16 and 24 per 10^4 g. of polypeptide; gram equivalents of *o*-chloro-anilides formed (by chlorine analysis), 10 and 11.5 per 10^4 g. of polypeptide; gram equivalent of acid groups lost during the reaction, 20 and 25 per 10^4 g. of polypeptide. The decrease in acid groups would be expected to be low if any of the anhydrides formed were hydrolyzable at *p*H 11.5.

Preparation of Polyglycine Esters.[27]—Freshly distilled glycine methyl ester was permitted to polymerize in ether solution at room temperature for one month. Glycine ethyl ester was held *in vacuo* at 70° for two weeks. The polymers (fractions A) were freed from glycine anhydride

(24) In contrast to the diketopiperazines derived from glycine and alanine, that from asparagine did not react with phenyl isocyanate, even at 150°, although this compound would be expected to bind four molecules—two on the amide groups and two on the ring peptide nitrogen groups.

(25) This compound was stable in 5 *N* hydrochloric acid for twenty-four hours at 120° but could be hydrolyzed with some discoloration at 150°. The protein-phenyl isocyanate derivatives also were markedly resistant to hydrolysis. Jensen and Evans (see footnote 15) demonstrated the stability of certain phenyl urea linkages in phenyl isocyanate-treated insulin.

Aniline, which is also a product of the hydrolysis, yielded no nitrogen under the conditions of the Van Slyke determination (three-minute reaction period).

(26) Hydrolyzed with 10 *N* hydrochloric acid at 150° for twenty four hours.

(27) Frankel and Katchalski, THIS JOURNAL, **64**, 2268 (1942).

and lower peptide esters by repeated extraction with boiling water. A second crop of highly polymerized material (B) was prepared from the cold water-soluble peptide esters of the first experiment (on the average hexapeptides) by heating these in the dry state to 135° for three weeks.

The extent of polymerization was gauged by amino nitrogen determinations, rather than by alkoxyl analysis.[27] Fraction A representing 12 and 28%, respectively, of the theoretical yield, was composed of an average of 15 and 16 glycine units; fraction B, comprising 19%, was composed of 72 units. The analytical data presented in Table IV show that fraction A corresponded in composition to a pure glycine polypeptide ester. On the other hand, fraction B, which was heated to 135° to achieve further polymerization, seemed to contain impurities.

Reaction of Isocyanates with Polyglycine Ester and Nylon.—A 0.4-g. sample of dry polyglycine ester (B) was treated with 2 ml. of phenyl isocyanate and 5 ml. of dry pyridine at 100° for two days. The extracted reaction product weighed 0.4 g. The nitrogen content was the same as that of the starting material (Table IV).

TABLE IV

POLYMERIZATION OF GLYCINE METHYL ESTER

	Fraction A		Fraction B	
	Found, %	Calculated for 15 units, %	Found, %	Calculated for 72 units, %
Free amino -N	1.60[a]	1.58	0.34	0.34
Amino-N liberated through acid hydrolysis	25.0	23.6	19.7	24.4
Total N	23.4	23.6	23.5	24.4

[a] A three-minute reaction period was used for all amino nitrogen analyses listed in this table; after a fifteen-minute reaction period, 1.68% was found.

A 0.5-g. sample of powdered Nylon, treated in the same manner with 1.0 ml. of *o*-chlorophenyl isocyanate and 3 ml. of pyridine yielded 0.49 g. of the extracted product. The chlorine content was 1.15% (3.3 equivalents per 10^4 g. of the material, originally containing 82 peptide bonds, and 0.14 terminal amino group).

We thank L. M. White of this Laboratory for the elementary analyses.

Summary

When proteins were treated with aromatic isocyanates under anhydrous conditions and in the presence of pyridine, reaction occurred with: (1) the basic groups, including amino, guanidyl, and imidazole; (2) the acid groups, including carboxyl, thiol and phenolic; and (3) the primary amide, and probably part of the aliphatic hydroxyl groups.

Under the same conditions the peptide groups of chain molecules did not react appreciably, in contrast to the simple diketopiperazines which bind two molecules of phenyl isocyanate.

The method described by Plimmer for quantitative liberation of the primary amide, amino, and the guanidyl nitrogen by the action of nitrous acid in mineral acid was applied successfully to proteins. This reaction made possible estimations of the extent of interaction of amide groups with phenyl isocyanate.

The products of the anhydrous reaction of phenyl isocyanate with alanine and with glycine and alanine anhydrides are described.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, LOS ANGELES]

Allylic Rearrangements. XVII. The Addition of the Butenyl Grignard Reagent to Diisopropyl Ketone[1]

BY WILLIAM G. YOUNG AND JOHN D. ROBERTS[2]

Preceding studies[3,4,5] have shown that the butenyl Grignard reagent reacts with carbon dioxide and simple aldehydes and ketones to give products corresponding exclusively to the secondary form of the Grignard reagent.

In order to test the generality of the appearance of the methylvinylcarbinyl radical in the products obtained from the reaction of the butenyl Grignard reagent with carbonyl compounds, it was desirable to investigate other more complicated cases, particularly those where it might be expected that steric influences would not favor the introduction of a secondary group. In this paper we present results obtained from the reaction of diisopropyl ketone with butenylmagnesium halides.

Whitmore and George[6] have made a thorough study of the addition of a number of aliphatic Grignard reagents to diisopropyl ketone. With increasing steric hindrance in the organomagnesium halide, the yield of addition products decreased and the competing reactions of enolization and reduction became increasingly important. With isopropylmagnesium bromide no addition product could be isolated and enolization and reduction took place to the extent of 29% and 65%, respectively. This result indicates that other larger (and more sterically hindered) secondary Grignard reagents would likewise give only enolization and reduction products.

From the foregoing it was anticipated that the reaction of diisopropyl ketone with butenylmagnesium halides should be found to follow either of two possible courses: (a) if the Grignard reagent can introduce only the secondary group into carbonyl compounds, then addition should

(1) Presented before the Division of Organic Chemistry at the New York meeting of the American Chemical Society, September, 1944.

(2) Abbott Laboratories Research Fellow, 1943-1944.

(3) Lane, Roberts and Young, THIS JOURNAL, 66, 543 (1944).

(4) Roberts and Young, *ibid.*, 67, 148 (1945).

(5) Ou Kuin-Houo, *Ann. chim.*, [11] 13, 175 (1940).

(6) Whitmore and George, THIS JOURNAL, 64, 1239 (1943); see also Conant and Blatt, *ibid.*, 51, 1227 (1929).