m.p. 163–164°, yield 1.5 g. Whether this derivative, and the one following, is still a mixture of racemic diastereomers or only a single racemic form due to fractionation, cannot be stated at this time.

Anal. Calcd. for $C_{12}H_{13}NO_6$: C, 53.9; H, 4.9; N, 5.2. Found: C, 53.9; H, 5.0; N, 5.2.

Carbobenzoxy-β-hydroxy-β-methylaspartic acid was prepared by carbobenzoxylation of the amino acid in the presence of excess sodium bicarbonate. The derivative, extracted from the acidified solution with ethyl acetate, was crystallized from chloroform, m.p. 154–156°. The compound is soluble in water, just as is its structural isomer, carbobenzoxy-γ-hydroxyglutamic acid.[4]

Anal. Calcd. for $C_{13}H_{15}NO_7$: C, 52.5; H, 5.1; N, 4.7. Found: C, 52.4; H, 5.1; N, 4.7.

Reduction of β-Hydroxy-β-methylaspartic Acid to β-Methylaspartic Acid with Hydriodic Acid.—Five grams of amino acid was refluxed 24 hr. in 250 ml. of 57% hydriodic acid containing 2 g. of red phosphorus. Paper chromatography of an aliquot, desalted by absorbing the amino acids onto Dowex 50 followed by elution with ammonium hydroxide, showed the reduction to be far from complete (less than 50%). The red phosphorus in suspension was filtered off, the hydriodic acid removed by distillation under vacuum, and the residue resubmitted to the same treatment as before. This was then repeated a third time after which the amount of hydroxyamino acid remaining was roughly estimated at about 20–30%. The mixture was filtered, repeatedly evaporated to dryness under vacuum, treated with silver carbonate to completely rid the solution of iodide, the solution filtered, treated with H_2S, filtered again, and the solution finally concentrated under vacuum to completely remove the H_2S. The residue was dissolved in 250 ml. of water and the solution boiled for 10 minutes in the presence of excess copper carbonate. The insoluble copper salt of the unchanged starting material thereby crystallized, while the much more soluble copper salt of the product remained in solution. The mixture was cooled several hours, filtered, and the filtrate freed of copper by H_2S treatment. The final solution was evaporated to an oil, the oil dissolved in a few ml. of water, the solution treated with charcoal and the amino acid crystallized by the addition of ethanol; yield 0.8 g. The product, recrystallized from water–ethanol, was chromatographically identical with a sample of authentic β-methylaspartic acid obtained by synthesis,[9,10] namely, R_f 0.44 in *sec*-butyl alcohol–formic acid–water (4:1:1) and 0.21 in phenol saturated with 10% sodium citrate.

Anal. Calcd. for $C_5H_9NO_4$: C, 40.8; H, 6.2; N, 9.5. Found: C, 40.9; H, 6.3; N, 9.3.

BETHESDA, MD.

[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, AND THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

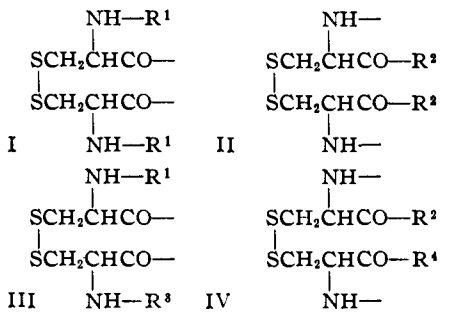
Preparation and Disulfide Interchange Reactions of Unsymmetrical Open-chain Derivatives of Cystine

BY LEONIDAS ZERVAS, LEO BENOITON, ELLINOR WEISS, MILTON WINITZ AND JESSE P. GREENSTEIN

RECEIVED OCTOBER 13, 1958

Preparation of mono-(carbobenzoxyglycyl)-L-cystine was achieved by treatment of an excess of L-cystine in aqueous alkali with carbobenzoxyglycyl chloride. Decarbobenzoxylation of the compound by the action of hydrogen bromide in glacial acetic acid permitted the subsequent isolation of pure, crystalline monoglycyl-L-cystine. The bis-methyl, bis-benzyl, monomethyl and monobenzyl ester derivatives of monocarbobenzoxy-L-cystine were prepared by the usual esterification procedures. Conversion of the first-mentioned derivative to N^{α}-carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine bis-methyl ester was effected by the action of trityl chloride in chloroform containing triethylamine. Treatment of such product with hydrazine did not lead to the expected α-monohydrazide but rather induced a rapid disulfide interchange with the formation of the symmetrical bis-trityl-L-cystine bis-methyl ester and bis-carbobenzoxy-L-cystine bis-hydrazide as the only isolable products. Comparable disulfide interchange of this same compound as well as of monocarbobenzoxy- and monoglycyl-L-cystine was catalyzed by alkali in both aqueous and methanolic solution. The rate and extent of such interchange in basic solution was shown to increase with an increase in *p*H.

Since substitution at both amino or both carboxyl functions of the symmetrical cystine molecule can involve either the same or different substituents, derivatives of this amino acid may be classified as symmetrical (I and II), unsymmetrical (III and IV) or combinations thereof. An impressive number of symmetrical open-chain peptides of cystine are presently accessible *via* a variety of synthetic routes,[1] whilst the synthesis of oxytocin, vasopressin and analogs thereof[2] has at the same time provided several unsymmetrical peptides of cystine wherein the disulfide linkage is implicated in a cyclic structure. In contrast, the development of methods which would permit the preparation of unsymmetrical open-chain derivatives of the type represented by structures (III) and (IV) has hitherto received only scant attention. Thus, an attempt by Fischer and Gerngross,[3] in 1909, to prepare monoglycyl- and monoleucyl-L-cystine through aminolysis of their respective mono-α-haloacyl-L-cystine precursors culminated in the isolation, in each instance, of a material which was presumed to be the desired product. Such presumption was nonetheless tinged with an



(1) *Cf.* J. S. Fruton, *Adv. in Protein Chem.*, **5**, 1 (1949); W. Grassmann and E. Wünsch, *Fortschr. Chem. org. Naturstoffe*, **13**, 445 (1956); M. Goodman and G. W. Kenner, *Adv. in Protein Chem.*, **12**, 465 (1957); J. P. Greenstein and M. Winitz "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., Vol. II, in press.

(2) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, THIS JOURNAL, **76**, 3115 (1954); V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, *ibid.*, **76**, 4751 (1954); P. G. Katsoyannis, *ibid.*, **79**, 109 (1957); C. Ressler and V. du Vigneaud, *ibid.*, **79**, 4511 (1957); V. du Vigneaud, M. F. Bartlett and A. Jöhl, *ibid.*, **79**, 5572 (1957).

(3) E. Fischer and O. Gerngross, *Ber.*, **42**, 1486 (1909).

element of doubt since the amorphous physical appearance of the isolated materials, as well as the marked deviation of their elemental analytical values from the calculated values, led to the admitted conclusion that each was grossly contaminated; that the synthesis did not rigorously follow the expected course was indicated by the fact that free cystine could be isolated from the reaction mixture in appreciable amount, a phenomenon which was ascribed to the hydrolysis of the reactant α-haloacylamino acid. No successful synthesis of an unsymmetrical open-chain peptide of cystine has been reported since this early but somewhat unrewarding effort. The present investigation was undertaken with the primary purpose of ultimately developing a method that would permit the synthesis of peptides of this latter type.

Results and Discussion

Preparation of Optically Pure L-Cystine.—For the synthesis of cystine derivatives employed in the present study, it was deemed essential that optically pure preparations of L-cystine be utilized as an initial reactant. Although the asymmetric hydrolytic action of hog renal acylase I on an epimeric mixture of the bis-acetyl derivatives of DL- and *meso*-cystine may be employed in the convenient preparation of several grams of optically pure L-cystine as well as its D- and *meso*-stereomers,[4] such method is not applicable for the purpose at hand since it does not permit the practicable preparation on a laboratory scale of the several hundred grams of L-cystine here required. Further, currently available commercial preparations of L-cystine do not suffice as these almost invariably reveal specific rotation values which are lower than the generally accepted value of −212° (in *N* HCl). Inasmuch as such commercial samples usually will react with D-amino acid oxidase but nonetheless possess elemental analytical values which are generally correct (if incorrect, the impurity which is generally tyrosine can be removed by crystallization procedures), it becomes plausible to assume that the oxidase reacting contaminant is, for the most part, a mixture of D- and *meso*-cystine. Since these latter compounds are oxidized quantitatively albeit slowly by D-amino acid oxidase, a possible approach to the elimination of such contaminants from commercial L-cystine preparations is afforded.

With the above in mind, Dr. W. K. Paik in this Laboratory developed a method for the large scale preparation of optically pure L-cystine. Toward this end, a sample of commercially-available L-cystine ($[\alpha]^{25}D$ −205° in 1 *N* HCl) was selected at random. To 300 mg. of this material, suspended in 1.5 ml. of 0.1 *M* sodium pyrophosphate buffer at *p*H 8.5 and 38° in a Warburg respirometer provided with a 20% potassium hydroxide solution in the center well, was added from the side-arm 0.5 ml. of a solution of 100 mg. of lyophilized renal D-amino acid oxidase. Oxygen consumption began immediately and was still continuing in nearly linear fashion after 7 hours, at which time nearly 5 μM. of oxygen had been absorbed; further incubation was deemed unnecessary. Instead, 100 g. of the L-cystine preparation was treated at *p*H 8.5 with renal D-amino acid oxidase in a manner analogous to that previously utilized[5] to secure a variety of optically pure L-amino acids from the corresponding racemates. Analytically pure L-cystine with an $[\alpha]^{25}D$ −215° (1% in 1 *N* HCl) was ultimately isolated from the digest in high yield; the oxidation by the enzyme of the D-cystine and of the D-half of the *meso*-cystine present evidently led to the formation of products soluble in the solvents employed. That the product possessed an optical purity which was greater than 99.9% was attested to upon its treatment with D-amino acid oxidase in the usual fashion.[6]

Disulfide Interchange in Unsymmetrical Open-chain Cystine Derivatives.—Recent studies[7-9] have demonstrated that disulfide interchange of cystine and its derivatives may occur in neutral, basic and strongly acidic solution. It was under such conditions that Ryle and Sanger[8] could demonstrate the formation of mono-(dinitrophenyl)-cystine, which was measured photometrically, from the interaction of cystine and bis-(dinitrophenyl)-cystine. These same investigators also established that the disulfide interchange between the last-mentioned compound and cystinyl-bis-glycine led to the production of mono-(dinitrophenyl)-cystinyl-glycine. The rate and extent of interchange in basic solution was shown to increase with an increase in *p*H. These findings, in addition to the results reported herein, tend to explain in part the unsatisfactory results obtained by Fischer and Gerngross[3] in their attempt to secure unsymmetrical peptides of cystine, as well as some of the difficulties which might be encountered during syntheses directed toward compounds of this type.

The key intermediate upon which the present study was based was monocarbobenzoxy-L-cystine (VI), formed by the interaction of a large excess of L-cystine (V) with carbobenzoxy chloride under Schotten–Baumann conditions; isolation of the monoacylated derivative required adjustment of the *p*H of the reaction mixture to 6, removal of the highly insoluble excess cystine by filtration, readjustment of the *p*H of the filtrate to 3.2, and recovery of the deposited monocarbobenzoxy-L-cystine.[4] Esterification of the latter product (VI) with methanolic HCl and with benzyl alcohol in the presence of *p*-toluenesulfonic acid yielded the corresponding bis-methyl ester hydrochloride (VII, R = CH_3, X = HCl) and bis-benzyl ester *p*-toluenesulfonate (VII, R = $CH_2C_6H_5$, X = *p*-$CH_3C_6H_4SO_3H$) derivatives, respectively.[10] Treat-

(4 R. Marshall, M. Winitz, S. M. Birnbaum and J. P. Greenstein, 1s JOURNAL, **79**, 4138 (1957).

(5) J. R. Parikh, J. P. Greenstein, M. Winitz and S. M. Birnbaum, *ibid.*, **80**, 953 (1958).

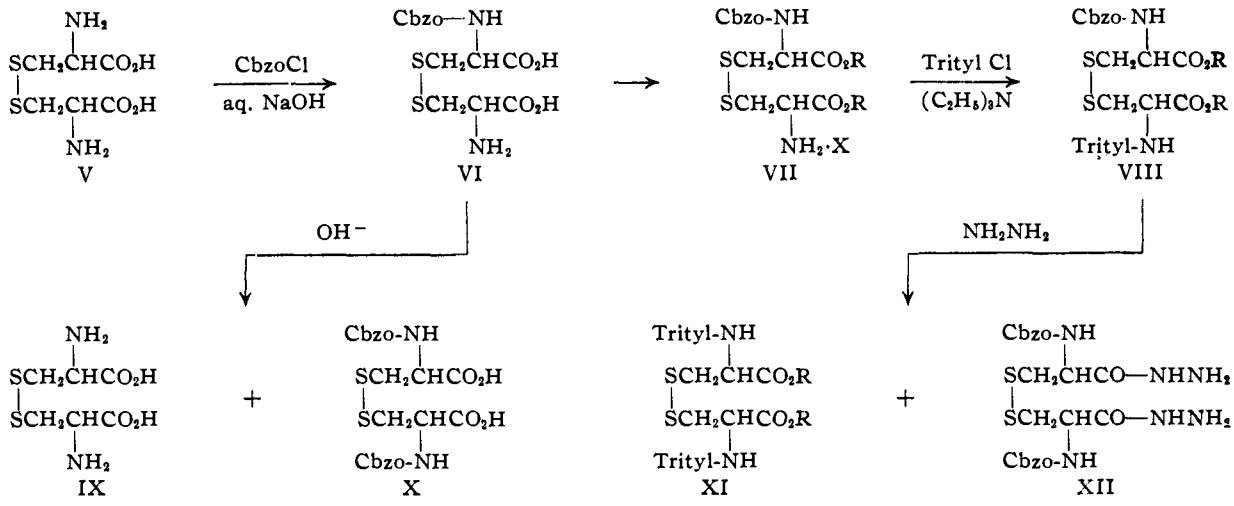
(6) A. Meister, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **192**, 535 (1951).

(7) F. Sanger, *Nature*, **171**, 1025 (1953).

(8) A. P. Ryle and F. Sanger, *Biochem. J.*, **60**, 535 (1955).

(9) R. E. Benesch and R. Benesch, THIS JOURNAL, **80**, 1666 (1958).

(10) Under controlled conditions, the monomethyl and monobenzyl ester derivatives of monocarbobenzoxy-L-cystine may also be procured. Since the monomethyl ester derivative liberates one mole of carbon dioxide as measured by the Van Slyke manometric ninhydrin-CO_2 procedure, and since it is susceptible to oxidative deamination by *Crotalus adamanteus* L-amino acid oxidase, the methoxycarbonyl group is presumably attached to the α-carbon atom which bears the N-acyl substituent. A like structure is here tentatively assigned to the monobenzyl ester derivative on the basis of analogy.



ment of VII with trityl chloride and triethylamine in chloroform solution[11] led, in turn, to the pertinent bis-ester of N^{α}-carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine (VIII). An attempt to prepare the corresponding α-monohydrazide by the interaction of VIII with several equivalents of hydrazine in methanolic solution proceeded, instead, with the rapid deposition of bis-trityl-L-cystine bis-ester[12] (XI, R = CH_3 or $CH_2C_6H_5$) in nearly quantitative amount; concentration of the mother liquors permitted the subsequent isolation of bis-carbobenzoxy-L-cystine bis-hydrazide (XII). The action of hydrazine had apparently triggered a disulfide rearrangement between two molecules of VIII to yield one molecule each of the symmetrical compounds XI and XII. That the reaction was base-catalyzed was evidenced by the fact that precipitation of the bis-trityl-L-cystine derivative XI could alternatively be induced by the addition of only a trace of either hydrazine or alkali to a methanolic solution of VIII. Analogous disulfide interchange was revealed by monocarbobenzoxy-L-cystine (VI) which, after treatment with diethylamine in aqueous solution for two hours, was converted to a mixture of free cystine (IX) and bis-carbobenzoxy-L-cystine (X) to the extent of some 40%; under the less alkaline conditions imposed by an aqueous solution of VI adjusted to *p*H 7.5 with sodium carbonate, some three days were required for only 25% of VI to undergo comparable rearrangement. On the other hand, no detectable interchange was noted at *p*H 6.5 after five days of storage. These results are not only reminiscent of the alkali-catalyzed disulfide interchange described by Ryle and Sanger[8] with the dinitrophenyl derivatives of cystine, but also explain the large amount of cystine formation noted by Fischer and Gerngross[3] during the aminolysis of mono-α-haloacylcystine. In any event, the findings indicate that the successful synthesis of unsymmetrical open-chain peptides of cystine is contingent upon the establishment of reaction conditions which would not be conducive to rearrangements about the disulfide linkage.

(11) L. Zervas and D. M. Theodoropoulos, THIS JOURNAL, **78**, 1359 (1956).

(12) No conversion of the ester moiety to a hydrazide group occurs under these conditions in consequence of the steric hindrance imposed by the bulky trityl substituent.[11]

Preparation of Monoglycyl-L-cystine.—As a first approach to the synthesis of unsymmetrical open-chain peptides of cystine, conditions comparable to those employed for the synthesis of monocarbobenzoxy-L-cystine were investigated. Thus, the treatment of an excess of L-cystine with carbobenzoxyglycyl chloride in aqueous dioxane under alkaline conditions permitted the ultimate isolation of pure mono-(carbobenzoxyglycyl)-L-cystine. Decarbobenzoxylation of the latter by the action of anhydrous hydrogen bromide in glacial acetic acid[13] yielded the desired glycyl-L-cystine in addition to a small amount of free cystine[14] which could be readily removed through fractional crystallization. Although such route made available for the first time a pure, crystalline unsymmetrical open-chain peptide of cystine, its application to the synthesis of peptides of more diverse structure is admittedly limited. Other approaches were therefore explored. Toward this end, an attempt was made to prepare monoglycyl-L-cystine *via* both the dicyclohexylcarbodiimide-[15] and the mixed anhydride-[16] mediated condensation of carbobenzoxyglycine with monocarbobenzoxy-L-cystine bis-benzyl ester, followed by removal of the blocking substituents from the intermediate N^{α}-carbobenzoxy-$N^{\alpha'}$-carbobenzoxyglycyl-L-cystine bis-benzyl ester with hydrogen bromide in glacial acetic acid.[13] Such effort culminated in the isolation of only small amounts of impure intermediate and final products. Alternative routes have not been hitherto investigated.

Experimental

I. **Stereochemical Purification of Commercial L-Cystine *via* the Action of D-Amino Acid Oxidase.**—One hundred

(13) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952); G. W. Anderson, J. Blodinger and A. D. Welcher, THIS JOURNAL, **74**, 5309 (1952); R. A. Boissonnas and G. Preitner, *Helv. Chim. Acta*, **36**, 875 (1953); D. Ben-Ishai, *J. Org. Chem.*, **19**, 62 (1954).

(14) Such occurrence provides another example of disulfide interchange in strongly acidic solution. This reaction might perhaps be avoided by using *p*-toluenesulfonic acid as the decarboxylation agent (*cf.* E. Taschner and B. Liberek, IVth International Congress of Biochemistry, Supplement to International Abstracts of Biological Sciences, Vienna, 1958, p. 1.

(15) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955); J. C. Sheehan, M. Goodman and G. P. Hess, *ibid.*, **78**, 1367 (1956).

(16) J. R. Vaughan and R. L. Osato, *ibid.*, **74**, 676 (1952); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951).

grams of the L-cystine sample ($[\alpha]^{25}D$ −205° in 1 *N* HCl) is suspended in 3 liters of water and just brought into solution by the addition of 2 *N* NaOH. The *p*H is adjusted to 8.5 by treatment with 2 *N* HCl and the total volume brought to 4 liters with water. To this mixture is added 2 liters of 0.33 *M* sodium pyrophosphate buffer at *p*H 8.5 and the whole mixed; some cystine precipitates at this point, but this is unimportant since the crystals are now "wet." About 10.5 g. of lyophilized, soluble hog kidney D-amino acid oxidase powder[5] is added and stirred to solution. A few ml. of octyl alcohol is added and a fairly rapid stream of oxygen gas is bubbled through the reaction mixture, with stirring, for a period of 4 days at 38°. At the end of this time, an additional 13.8 g. of the enzyme powder is added to the digest and the oxygenation continued for another 2 days as above. Thereafter, the *p*H of the reaction mixture is adjusted to *p*H 5 by the addition of 2 *N* HCl, and the combined precipitate of L-cystine and protein admixed with 5 g. of pure charcoal and filtered off. The precipitate is washed well with water and extracted twice with 2 *N* HCl at 70° (1 liter of 2 *N* HCl at 70° each time). Upon chilling the combined clear filtrates and adjusting the *p*H thereof to 5 by the addition of dilute ammonium hydroxide, crystallization of the L-cystine ensues. After storage in the cold for several hours, the precipitate is recovered by filtration, re-dissolved in HCl, and the solution filtered after the addition thereto of a few grams of charcoal. The clear filtrate is again neutralized to *p*H 5 as before, chilled, and the precipitate of optically-pure L-cystine recovered by filtration and washed successively with large amounts of water, ethanol and ether; yield 85 g., $[\alpha]^{25}D$ −215° (1% in 1 *N* HCl).

Treatment of 300 μM. of the above product with D-amino acid oxidase in the Warburg respirometer leads to the consumption of less than 0.1 μM. of oxygen in 7 hours.[6] As the subsequent addition thereto of 0.3 μM. of D-cystine results in a quantitative oxygen uptake, the L-cystine may be considered to possess an optical purity which is greater than 99.9%.

II. Preparation and Disulfide Interchange of Monocarbobenzoxy-L-cystine.—This compound was prepared as described previously[4] except for the following minor modifications employed during the purification stage. Thus, after the carbobenzoxylation of 100 g. of L-cystine, acidification of the reaction mixture to *p*H 6, and removal of the free cystine by filtration, the crude monocarbobenzoxy-L-cystine is precipitated from the cooled filtrate by adjustment of the *p*H to 3.2 with HCl. The product secured after filtration is washed successively with ethanol and ether, and is then suspended and shaken in 100 ml. of 1 *N* HCl for 1 hour in order to remove the final traces of free cystine. Following recovery of the insoluble product by filtration, it is dissolved in the required amount of aqueous diethylamine (15 g. per 300 ml.) and the solution treated immediately with an excess of acetic acid. Crystallization begins after a few minutes and the deposit is collected by filtration after storage for several hours in the cold. The product is obtained in a 15 g. yield after washing first with ethanol, then with ether and drying *in vacuo;* the $[\alpha]^{23}D$ value of −117.8° (1% in 1 *N* sodium hydroxide) is in agreement with the previously observed value[4] of −120° under the same conditions.

The following experiments reveal the tendency of monocarbobenzoxy-L-cystine to undergo disulfide interchange under alkaline conditions: (a) A mixture of 3.7 g. of monocarbobenzoxy-L-cystine, 4 ml. of water, 4.5 ml. of diethylamine and 8 ml. of isopropyl alcohol is shaken at room temperature for 2 hours and 20 ml. of 1 *N* NaOH then added thereto. After 15 minutes, the reaction mixture is acidified to *p*H 2 with HCl and the resulting suspension first extracted with ethyl acetate and then filtered to give 1.6 g. of the unchanged monocarbobenzoxyamino acid. The aqueous filtrate is adjusted to *p*H 6 with sodium hydroxide, cooled and filtered; crystallization of the precipitate from HCl solution by neutralization with alkali yields 200 mg. of L-cystine. The above ethyl acetate extracts are dried over anhydrous sodium sulfate and concentrated *in vacuo* to a residual material which is crystallized first from a chloroform–petroleum ether mixture and subsequently from chloroform to yield 1 g. of bis-carbobenzoxy-L-cystine[17] m.p. 121°. Such results lead to the conclusion that a minimum of some 40% disulfide interchange has taken place under these conditions.

(17) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(b) A solution of 3.7 g. of monocarbobenzoxy-L-cystine is adjusted to *p*H 7.5 with sodium carbonate and stored for 3 days at room temperature. Recovery of the bicarbobenzoxy-L-cystine as in (a) above yields 600 mg. of material. This corresponds to an interchange of some 24%.

(c) In an analogous experiment to that given under (b) above, 3.7 g. of monocarbobenzoxy-L-cystine in phosphate buffer at *p*H 7.5 is stored at room temperature for 16 hours. The consequent yield of bis-carbobenzoxy-L-cystine is 200 mg., which corresponds to an 8% interchange.

(d) Storage of a solution of monocarbobenzoxy-L-cystine at *p*H 6.5 for 5 weeks at room temperature leads to no appreciable formation of interchange products.

II. Preparation and Disulfide Interchange of the Ester Derivatives of N^{α}-Carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine. The Monocarbobenzoxy-L-cystine Bis-methyl Ester Hydrochloride.—A solution of 18.7 g. of monocarbobenzoxy-L-cystine in 50 ml. of cold 2 *N* methanolic HCl is permitted to stand at room temperature for one day and then concentrated to dryness *in vacuo* at 40°. The evaporation is repeated twice after the addition each time of 25 ml. of methanol. The residue thereby obtained is dissolved in 50 ml. of cold 1 *N* methanolic HCl and the entire procedure repeated. Solution of the final residual material in a little methanol, followed by the addition of acetone thereto, leads to the precipitation of the desired compound as colorless needles; yield 74%, m.p. 159–160°, $[\alpha]^{25}D$ −82.5° (1% in methanol).

Anal. Calcd. for $C_{16}H_{22}N_2O_6ClS_2$: C, 43.8; H, 5.3; N, 6.4; Cl, 8.1; methoxyl, 14.1. Found: C, 43.7; H, 5.3; N, 6.6; Cl, 8.1; methoxyl, 14.1.

N^{α}-Carbobenzoxy-L-cystine α-Methyl Ester.—A solution of 1.86 g. of monocarbobenzoxy-L-cystine in 23.5 ml. of 0.213 *N* methanolic HCl is stored at room temperature for 3 days, after which time no starting material remained as indicated by paper chromatography. Treatment of the reaction mixture with an excess of pyridine is followed by evaporation of the solvent *in vacuo*. Upon the addition of water to the residual material, crystals appear which are recovered by filtration and recrystallized from water; yield 1 g., $[\alpha]^{25}D$ −232.2° (0.8% in methanol containing 1 equivalent of HCl).

Anal. Calcd. for $C_{15}H_{20}N_2O_6S_2 \cdot {}^1/_2H_2O$: C, 45.3; H, 5.3; N, 7.0. Found: C, 45.7; H, 5.4; N, 6.8.

The anhydrous material is obtained by drying at 78° *in vacuo* over phosphorus pentoxide.

Anal. Calcd. for $C_{15}H_{20}N_2O_6S_2$: C, 46.4; H, 5.2; N, 7.2. Found: C, 46.4; H, 5.2; N, 7.2.

Monocarbobenzoxy-L-cystine Bis-benzyl Ester *p*-Toluenesulfonate.—A mixture of 7.5 g. of monocarbobenzoxy-L-cystine, 4.0 g. of *p*-toluenesulfonic acid monohydrate, 20 ml. of benzyl alcohol and 100 ml. of benzene is heated under reflux for 3 hours, with the water liberated during the reaction being removed azeotropically with the aid of a Dean and Stark distilling receiver.[18] After cooling, the reaction mixture is treated with several volumes of ether with the concomitant separation of an oil, which crystallizes upon scratching. The crystals are filtered off and recrystallized from ethyl acetate; yield 10.1 g., m.p. 133–134° (with sintering at 100°), $[\alpha]^{25}D$ −37.0° (1% in acetone).

Anal. Calcd. for $C_{35}H_{38}N_2O_9S_3$: C, 57.8; H, 5.3; N, 3.9. Found: C, 57.3; H, 5.3; N, 3.9.

N^{α}-Carbobenzoxy-L-cystine α-Benzyl Ester.—A mixture of 3.8 g. of anhydrous *p*-toluenesulfonic acid, 7.5 g. of monocarbobenzoxy-L-cystine and 100 ml. of benzyl alcohol is stored at room temperature for 9 days. The addition of several volumes of ether leads to the formation of a precipitate which is recovered by filtration and washed copiously by trituration with water; yield 8 g. Recrystallization from methanol yields the monohydrate; $[\alpha]^{25}D$ −191.2° (0.5% in methanol containing 1 equivalent of HCl).

Anal. Calcd. for $C_{21}H_{24}N_2O_6S_2 \cdot H_2O$: C, 52.5; H, 5.5; N, 5.8. Found: C, 52.1; H, 5.5; N, 5.9.

The anhydrous material results after drying at 78° *in vacuo* over phosphorus pentoxide.

Anal. Calcd. for $C_{21}H_{24}N_2O_6S_2$: C, 54.5; H, 5.2; N, 6.1. Found: C, 54.2; H, 5.4; N, 6.0.

(18) *Cf.* L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).

N^{α}-Carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine Bis-methyl Ester.—To a suspension of 4.4 g. of monocarbobenzoxy-L-cystine bismethyl ester hydrochloride in 30 ml. of anhydrous chloroform is added 3.0 ml. of pure anhydrous triethylamine.[11] After the ester has dissolved, 2.8 g. of trityl chloride is added and the solution stored overnight at room temperature. The reaction mixture is washed successively with water and chloroform and then evaporated *in vacuo*. After the addition of a little methanol to the residual material, the evaporation process is repeated. A residual sirup is obtained which crystallizes in the presence of methanol upon scratching with a glass rod. Recrystallization is effected from methanol; yield 75%, m.p. 66–67°, $[\alpha]^{25}D$ +103° (1% in chloroform).

Anal. Calcd. for $C_{35}H_{30}O_6N_2S_2$: C, 65.2; H, 5.6; N, 4.3. Found: C, 64.9; H, 5.7; N, 4.2.

Detritylation of sample of the aforementioned compound was effected as follows: A solution of 0.64 g. of N^{α}-carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine bis-methyl ester in 2 ml. of 0.5 *N* methanolic HCl is boiled for 1–2 minutes.[11] On the addition of ether to the cooled reaction mixture, monocarbobenzoxy-L-cystine bis-methyl ester hydrochloride precipitates in nearly quantitative yield, m.p. 159–160°, $[\alpha]^{25}D$ −82.5° (1% in methanol). The melting point remains unchanged upon admixture with an authentic sample of monocarbobenzoxy-L-cystine bismethyl ester hydrochloride.

The preparation of the bis-benzyl ester of N^{α}-carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine from 1.45 g. of monocarbobenzoxy-L-cystine bis-benzyl ester *p*-toluenesulfonate, 0.6 ml. of triethylamine and 0.56 g. of trityl chloride in 10 ml. of chloroform was attempted by a procedure analogous to that employed to procure the corresponding dimethyl ester. An oil ensued which resisted crystallization.

Bis-carbobenzoxy-L-cystine Bis-hydrazide.—A solution of 5.08 g. of bis-carbobenzoxy-L-cystine in 10 ml. of 2 *N* methanolic HCl is stored at room temperature overnight and the solvent then evaporated *in vacuo*. The evaporation is repeated twice after the addition each time of a small amount of absolute methanol. The residual material is treated with 10 ml. of 1 *N* methanolic HCl, the reaction mixture permitted to stand overnight, and the evaporation of the solution effected as above. An oil results which is dissolved in 80 ml. of methanol to which 1.3 ml. of hydrazine hydrate has been added. Upon storage of the solution overnight at room temperature, a precipitate forms which is recovered by filtration and recrystallized from ethanol; yield 3.1 g., m.p. 175–176°.

Anal. Calcd. for $C_{22}H_{28}O_6N_6S_2$: C, 49.2; H, 5.3; N, 15.7. Found: C, 49.4; H, 5.4; N, 15.5.

Disulfide Interchange Reactions.—Treatment of N^{α}-carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine bis-methyl ester with hydrazine in methanol does not yield the anticipated N^{α}-carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine monomethyl ester monohydrazide but proceeds instead with the formation of two reaction products, bis-trityl-L-cystine bis-methyl ester and bis-carbobenzoxy-L-cystine bis-hydrazide. This indicates the occurrence of an interchange at the disulfide linkage under the reaction conditions employed. Comparable dismutation is mediated in alkaline media. Such phenomenon is demonstrated by the experiments which follow: (a) A solution of 1.9 g. (0.003 mole) of N^{α}-carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine bis-methyl ester in 30 ml. of methanol is treated with 2 ml. of hydrazine hydrate and the mixture permitted to stand at room temperature overnight. A precipitate forms which is filtered over suction, washed with methanol and then recrystallized from methanol; yield 0.9 g., m.p. 145–146°. No depression is shown by a mixed melting point with an authentic sample of bis-trityl-L cystine bis-methyl ester.[11] As a further proof of its structure, detritylation of 0.35 g. of the compound by treatment with 4 ml. of boiling 0.5 *N* methanolic HCl for 1 minute yields a product, m.p. 170°, whose mixed melting point with L-cystine bis-methyl ester dihydrochloride[19] is without depression. From the concentrated mother liquors of the original reaction mixture are obtained two crystalline fractions, namely, 0.4 g. of a material melting at 139–140° and 0.3 g. of a material melting at 125°. Washing of the lower melting fraction thoroughly with benzene, in which bis-trityl-L-cystine bis-methyl ester is quite soluble, results in a marked elevation of the melting point to 170–171°. A mixed melting point with bis-carbobenzoxy-L-cystine bis-hydrazide is without depression.

(b) To a solution of 1.1 g. of N^{α}-carbobenzoxy-$N^{\alpha'}$-, trityl-L-cystine bis-methyl ester in 25 ml. of methanol is added 0.2 g. of hydrazine hydrate and the reaction mixture stored overnight at room temperature. After this time, 0.6 g. of a solid deposit is recovered by filtration. Extraction of the precipitate with benzene leaves only 0.05 g. of an insoluble residue. Evaporation of the benzene extracts yields 0.55 g. of bis-trityl-L-cystine bis-methyl ester, m.p. 143–144°. Concentration of the original mother liquors yields a residue which is washed with benzene. The combined benzene-insoluble fractions, after crystallization from ethanol, yield 0.30 g. of bis-carbobenzoxy-L-cystine bis-hydrazide, m.p. 169–170°.

(c) A mixture of 1.9 g. of N^{α}-carbobenzoxy-$N^{\alpha'}$-trityl-L-cystine bis-methyl ester, 30 ml. of methanol and 0.3 ml. of 1 *N* methanolic KOH is allowed to stand at room temperature for 2 days. The colorless precipitate which forms is filtered off and recrystallized from methanol; yield 0.28 g., m.p. 146–147°. A mixed melting point with bis-trityl-L-cystine bis-methyl ester is without depression. No saponification occurs during the aforementioned process by virtue of the steric hindrance afforded by the bulky trityl substituent.[11]

IV. Preparation and Disulfide Interchange of Monoglycyl-L-cystine. Mono-(carbobenzoxyglycyl)-L-cystine.—Solid carbobenzoxyglycyl chloride[17] (prepared from 10.5 g. of carbobenzoxyglycine) is added in four equal portions, with vigorous shaking, to a solution of 36.0 g. (0.15 mole) of L-cystine in a mixture of 95 ml. of 4 *N* sodium hydroxide and 95 ml. of dioxane at 0°. The *p*H of the solution is adjusted to 6 with hydrochloric acid within 10 minutes after the initial addition of acid chloride, and the dioxane is subsequently removed from the reaction mixture by evaporation under reduced pressure. After treatment of the concentrate with 50 ml. of water, the cystine is filtered off with the help of a filter aid and the filtrate adjusted to *p*H 1.8 with concentrated hydrochloric acid.[20] The mixture is then washed twice with ethyl acetate and the aqueous fraction carefully brought to *p*H 3.2 with lithium hydroxide solution. The resulting solution (approximately 150 ml.), after storage in the cold overnight, yields 5.5 g. of the desired product, which is collected over suction and washed first with cold water, then with ethanol; an additional 1.0 g. of product is recovered by concentration of the filtrate, followed by the addition of ethanol thereto. Recrystallization is accomplished from water by the addition of ethanol; m.p. 178–180° dec., $[\alpha]^{25}D$ −136° (2% in water containing 1 equivalent of hydrochloric acid).

Anal. Calcd. for $C_{16}H_{21}N_3O_7S_2$: C, 44.4; H, 5.1; N, 9.7; S, 14.8. Found: C, 44.2; H, 5.1; N, 9.8; S, 15.0.

Monoglycyl-L-cystine.—Three grams of finely pulverized mono-(carbobenzoxyglycyl)-L-cystine is treated with a 22% solution of dry hydrogen bromide in glacial acetic acid and the insoluble material triturated with a glass rod. After about an hour at room temperature, an additional 10 ml. of the hydrogen bromide–glacial acetic acid reagent is added and the mixture stored for a further 2 hours. The reaction mixture is then treated with several volumes of absolute ether and the insoluble deposit washed copiously with ether and finally filtered. The precipitate is dissolved in 25 ml. of water and the solution adjusted to *p*H 6.2 with diethylamine. After cooling overnight, 200 mg. of cystine is recovered by filtration. Addition of ethanol to the filtrate yields a crystalline precipitate which is recrystallized twice from water–ethanol and air-dried; yield 1.0 g., m.p. 180–181° dec., $[\alpha]^{25}D$ −189° (1% in water). The analytical data indicates that each molecule of peptide is associated with 1.5 molecules of water of hydration.

Anal. Calcd. for $C_8H_{15}N_3O_5S_2 \cdot 1.5H_2O$: C, 29.6; H, 5.6; N, 12.9; S, 19.8. Found: C, 29.4; H, 5.8; N, 12.9; S, 19.8.

Upon drying *in vacuo* over phosphorus pentoxide for 3 hours at 78°; a 9% loss in weight occurs to yield the anhy-

(19) E. Fischer and U. Suzuki, *Z. physiol. Chem.*, **46**, 406 (1905).

(20) The volume of the filtrate is kept between 300 to 400 ml. in order to preclude the possibility of precipitation of the desired compound during the acidification; it is also helpful to add a small amount of ethyl acetate to the reaction mixture just prior to the acidification step.

drous material. The anhydrous material shows a tendency to be hygroscopic.

Anal. Calcd. for $C_8H_{15}N_3O_6S_2$: C, 32.3; H, 5.1; N, 14.2; S, 21.6. Found: C, 32.4; H, 5.1; N, 14.1; S, 21.3.

Disulfide Interchange.—A solution of 600 mg. of glycyl-L-cystine in water is adjusted to *p*H 7.5 with sodium carbonate and the total volume brought to 20 ml. After storage at 25° for 7 days, the *p*H is adjusted to 6, the reaction mixture concentrated *in vacuo*, and the precipitated cystine collected; yield 50 mg. This corresponds to a disulfide interchange of the order of 20%.

ATHENS, GREECE
BETHESDA, MD.

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Purine N-Oxides. V. Oxides of Adenine Nucleotides[1]

BY MARCUS A. STEVENS, HERMAN W. SMITH AND GEORGE BOSWORTH BROWN

RECEIVED AUGUST 21, 1958

The 1-N-oxides of adenosine 2′-, 3′- and 5′-phosphates and of adenosine 5′-diphosphate have been prepared from the parent nucleotides by oxidation with hydrogen peroxide–acetic acid mixtures. Oxidation with hydrogen peroxide alone has been used to prepare chromatographic quantities of the 1-N-oxides of adenosine 5′-monophosphate and deoxyadenylic acid. These nucleotide oxides have been characterized by hydrogenation to the parent nucleotide and by acid hydrolysis to 4-aminoimidazole-5-carboxamidoxime. Alkaline hydrolyses of adenosine 1-N-oxide and of the adenosine 2′-, 3′- and 5′-phosphate 1-N-oxides give the 1-ribosyl and 1-phosphoribosyl derivatives of 5-aminoimidazole-4-carboxamidoxime, respectively. The nucleotide oxides in the solid state and in aqueous solution exhibit an instability which is not observed with adenine 1-N-oxide or adenosine 1-N-oxide.

The preparation of mono-N-oxides of adenine and adenosine and the characterization of these oxides as 1-oxides has been reported.[2,3] In the hope of preparing a material which may be an effective antimetabolite for normal nucleotides, or have significance in biological oxidation–reduction systems, the series has been extended to the adenine nucleotide 1-N-oxides.[4] It has been found possible to prepare such oxides in ways similar to those used for the oxidation of adenine and adenosine. Oxidation of AMP-5′ for 16 days with a mixture of acetic acid and hydrogen peroxide gives the 1-N-oxide in a yield of 62%. Under certain conditions, AMP-5′ 1-N-oxide precipitates directly from the oxidizing solution, but the yield is variable. More reproducible yields are obtained when the AMP-5′ 1-N-oxide is separated from the unoxidized AMP-5′ by chromatography on Dowex-1-formate with 0.1 *M* formic acid.[5]

Either AMP-2′ or AMP-3′ gives a mixture of the 1-N-oxides of AMP-2′ and AMP-3′ upon oxidation with acetic acid and hydrogen peroxide. The mixture obtained by oxidizing AMP-3′ for a certain period of time appears to be identical in composition with that obtained from AMP-2′ by oxidation for the same period of time. Equilibration of the 2′-phosphate and 3′-phosphate forms must be occurring in the acetic acid medium used, though it may be that the AMP-2′ and -3′ 1-N-oxides also equilibrate. This type of equilibration was observed by Brown and Todd,[6] with the 2′,3′-phosphodiester proposed as the intermediate. When attempts were made to isolate the separate nucleotides from the mixture of nucleotides produced by the oxidation of either AMP-2′ or AMP-3′ by gradient elution with formic acid from a Dowex-1-formate column, it was found possible to separate the AMP-2′ and AMP-2′ 1-N-oxide from each other, but complete separation of the AMP-3′ from the AMP-3′ 1-N-oxide was not found possible. To circumvent this problem a sample of AMP-3′ (or AMP-2′) was oxidized until it had been largely converted to a mixture of oxides; then separation on a column was carried out by direct elution with 0.1 *N* formic acid. This yielded a little AMP-2′ (1st peak, small amount), AMP-2′ 1-N-oxide (2nd peak, large amount) and AMP-3′ 1-N-oxide (3rd peak, large amount + small amount of AMP-3′). An essentially pure sample of AMP-3′ 1-N-oxide was obtained from this last peak by taking the solution representing the first two thirds of the peak, evaporating to dryness and recrystallizing from acetone–water. After oxidation with an acetic acid–hydrogen peroxide mixture at 25° for 9 days, AMP-2′ 1-N-oxide was obtained in 17.4% yield, and AMP-3′ 1-N-oxide (partially contaminated with AMP-3′) was obtained in 25.4% yield.

ADP is also oxidized by hydrogen peroxide–acetic acid to a 1-N-oxide, but chromatography was not a satisfactory method of isolating the product. Fractional precipitation of the barium salt was used to give virtually pure ADP 1-N-oxide (as barium salt). ATP and deoxyAMP decompose to adenine when attempts are made to oxidize them with mixtures of acetic acid–hydrogen peroxide at room temperature.

The structures of the products obtained by the oxidation of AMP-2′, 3′ and -5′, and of ADP have been determined from elemental analysis, by reduction to the unoxidized nucleotide with hydrogen and Raney nickel catalyst and by hydrolysis to adenine 1-N-oxide (II) or to 4-aminoimidazole-5-carboxamidoxime (III). They are all 1-N-oxides. The reactions used for characterization are il-

(1) This investigation was supported in part by funds from the American Cancer Society (Grant #MET-27), National Cancer Institute, National Institutes of Health, Public Health Service (Grant #CY-3190) and from the Atomic Energy Commission (Contract #AT930-1-910).

(2) M. A. Stevens, D I. Magrath, H. W. Smith and G. B. Brown, THIS JOURNAL, **80**, 2755 (1958).

(3) M. A. Stevens and G. B. Brown, *ibid.*, **80**, 2759 (1958).

(4) The following abbreviations are used: AMP-2′, -3′ and -5′ for the 2′-, 3′- and 5′-phosphates of adenosine, respectively, ADP and ATP for adenosine 5′-diphosphate and 5′-triphosphate, and deoxyAMP for 2′-deoxyadenosine 5′-phosphate.

(5) W. E. Cohn, THIS JOURNAL, **72**, 1171 (1950).

(6) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952).