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Studies on Polycysteine Peptides and Proteins. III. Configurations of the Peptides of L-Cystine Obtained by Oxidation of L-Cysteinyl-L-cysteine

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The oxidation of L-cysteinyl-L-cysteine has been investigated by aeration in aqueous solution over the *p*H range 4.5–10.7 and at concentrations of 0.015–3.0%. Paper chromatographic analysis indicated a mixture of at least three ninhydrin-reactive components. The proportion of low R_f (phenol) (0.00–0.15), high molecular weight material was found to be lower in the products of oxidation carried out at the more dilute levels. The proportion of the other two components varied with the *p*H of the solution during oxidation. Outside the *p*H range 7.0–9.6 the formation of high R_f (0.49) component was favored almost to the exclusion of the medium R_f (0.18) component. The latter, however, was formed in appreciable quantities within this *p*H range. Molecular weight determinations by a new ultracentrifuge technique developed by Kegeles on the pure high R_f (0.49) component showed this to be the cyclic monopeptide to which the designation "cyclo-L-cystinyl" has been given. Similar determinations on mixtures of the R_f 0.18 and 0.49 components indicated that the R_f 0.18 component is very probably dimeric. Application of the dinitrophenyl technique to the study of these compounds demonstrated that this dimeric component consists largely of "parallel" cystinylcystine, but the possibility of the presence, in addition, of the "antiparallel" isomer cannot be excluded.

The importance of cyclic structures in proteins and large peptides has been strikingly emphasized in recent years by the work of Sanger on insulin,[1] and of du Vigneaud and co-workers on oxytocin and vasopressin.[2] Each of these compounds contains peptide chains joined into ring structures by the disulfide bridges of component cystine residues. Work on compounds of this type, containing only cystine residues was first carried out in 1937 by one of the present authors, when the oxidative conversion of L-cysteinyl-L-cysteine to L-cystinyl-L-cystine was described.[3] This conversion was conducted at a weakly alkaline reaction, and was terminated when the nitroprusside reaction of the solution became negative. The crystalline, oxidized peptide possessed an $[\alpha]^{22}D$ −60° (*c* 1, 1 *N* HCl) and crystallized from water as the dihydrate. On HCl hydrolysis, the peptide yielded nearly the equivalent amount of L-cystine. Molecular weight determinations of the N,N-dibenzoyldimethyl ester derivative gave values close to that calculated for the dimer. The course of the oxidative reaction may be represented as

$$\underset{\text{I}}{SHCH_2CH(NH_2)CONHCH(COOH)CH_2SH} \xrightarrow[\text{weakly alkaline reaction}]{O_2}$$

$$\begin{array}{l} SCH_2CH(NH_2)CONHCH(COOH)CH_2S \\ | \qquad\qquad\qquad\qquad\qquad\qquad | \\ SCH_2CH(NH_2)CONHCH(COOH)CH_2S \end{array} \quad \text{and/or}$$

II

$$\begin{array}{l} SCH_2CH(NH_2)CONHCH(COOH)CH_2S \\ | \qquad\qquad\qquad\qquad\qquad\qquad | \\ SCH_2CH(COOH)NHCOCH(NH_2)CH_2S \end{array}$$

III

whereby the oxidation of L-cysteinyl-L-cysteine (I) may form L-cystinyl-L-cystine of either the "parallel" (II) or the "antiparallel" (III) variety, or a mixture of both. No means were available at the time to decide between these alternatives, nor to determine whether other polymeric structures were simultaneously formed during the oxidative reaction and subsequently removed during the purification stages.

The development of new experimental techniques, together with the significant findings on the structures of insulin and of oxytocin alluded to above, prompted a resumption of this investigation. Four preparations of L-cystinyl-L-cystine obtained by oxidation of L-cysteinyl-L-cysteine at *p*H 8.5 possessed $[\alpha]^{26}D$ values, respectively, of −58.7°, −56.2°, −52.7°, and −46.0° (all at *c* 1, 1 N HCl).[4] Each of the four preparations showed two ninhydrin spots on the paper chromatogram (phenol), one a major spot with R_f = 0.18, the other a relatively minor spot with R_f = 0.49. The intensity of the latter spot varied inversely with the magnitude of the $[\alpha]D$ values of the preparations. The nature of this minor contaminant was suggested when the oxidation of L-cysteinyl-L-cysteine was conducted at *p*H 6.5; two preparations of the oxidized peptide formed under these conditions gave $[\alpha]^{26}D$ values, respectively, of −31.0° and −32.1° (both at *c* 1, 1 *N* HCl), and each preparation gave a single ninhydrin spot on the paper chromatogram with R_f = 0.49 (phenol). Whether formed at *p*H 8.5 or at 6.5, the peptides of L-cystine yielded on HCl hydrolysis pure L-cystine in good yield. There seems little doubt but that the oxidation of L-cysteinyl-L-cysteine at *p*H values as different as 6.5 and 8.5 leads to different peptides of L-cystine, that at *p*H 6.5 being a pure component, and that at *p*H 8.5 being contaminated to a greater or lesser degree with the form ordinarily obtained at *p*H 6.5.[5] The attempt to distinguish these different forms is the purpose of the present communication.

Results and Discussion

Effect of Concentration.—The present investigation began with a study of the nature of the prod-

(1) A. P. Ryle and F. Sanger, *Biochem. J.*, **60**, 535 (1955); A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *ibid.*, **60**, 541 (1955); H. Brown, F. Sanger and R. Kitai, *ibid.*, **60**, 556 (1955).

(2) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, THIS JOURNAL, **75**, 4879 (1953).

(3) J. P. Greenstein, *J. Biol. Chem.*, **118**, 321 (1937); **121**, 9 (1937).

(4) N. Izumiya and J. P. Greenstein, *Archiv. Biochem. Biophys.*, **52**, 203 (1954).

(5) A quite different picture emerges on oxidation of L-cysteinyl-D-cysteine or D-cysteinyl-L-cysteine at these two *p*H values.[4] The corresponding peptides of cystine, except for their optical enantiomorphism, appeared to be identical, and independent of the *p*H at which they were formed. They crystallized from water with no evidence of water of crystallization, yielded a single ninhydrin spot on the paper chromatogram with R_f = 0.16 (phenol) for each, and on HCl hydrolysis gave good yields of DL-cystine.

ucts formed by oxidation of L-cysteinyl-L-cysteine at *p*H 6.5 and at *p*H 8.5 in aqueous solutions of differing concentration. For this purpose, the solutions were subjected to paper chromatography using a phenol solvent. In every case a mixture of components separated on the chromatograms which could be distinguished by three ninhydrin-reactive areas, namely (A) a fast-moving spot with R_f = 0.49, a slow-moving spot (B) with R_f = 0.18, and finally an indefinite streak (C) with R_f = 0.0–0.15, presumably composed of high molecular weight material. The relative intensity of these areas is described in Table I.[6]

TABLE I

RELATIVE INTENSITY OF NINHYDRIN-REACTIVE AREAS ON PAPER CHROMATOGRAMS OF SOLUTIONS AT DIFFERENT CONCENTRATIONS OF AIR-OXIDIZED L-CYSTEINYL-L-CYSTEINE[a]

Concn., %	At *p*H 6.5			At *p*H 8.5		
	A	B	C	A	B	C
0.015	....	....	....	Strong	Weak	Strong
0.15	Strong	Weak	Strong	Strong	Strong	Strong
0.6	Strong	Weak	Strong	Weak	Strong	Very strong
3.0	Absent	Strong	Very strong	Trace	Strong	Very strong

[a] Phenol solvent used for chromatograms: A = R_f 0.49, B = R_f 0.18, and C = R_f 0.0–0.15.

The high intensity of (C) under all conditions suggests that the mass of materials represented by this streak occurs in relatively large proportion. The R_f for L-cystine in the phenol solvent is 0.16, and if this compound were present in the 0.0–0.15 streak (C) it could not be readily distinguished by paper chromatography from the remainder of the material nor, for that matter, from the (B) spot with R_f = 0.18. Since it was desirable to assure the absence of L-cystine from the subsequently isolated (A) and (B) components, recourse was made to the use of other solvents for paper chromatography. The R_f values for the (A), (B) and (C) components and for pure L-cystine in a ketone mix[7] were, respectively, 0.74, 0.55, 0.0–0.25 and 0.27. In a phenol–citrate buffer,[8] these R_f values were, respectively, 0.40, 0.26, 0.0–0.14 and 0.06. With these solvent mixtures, the absence of L-cystine from the (A) and (B) components could be assured, but the differences in R_f between the (A) and (B) spots were not as great as in the phenol solvent.

Oxidation of L-cysteinyl-L-cysteine in increasingly more concentrated solutions at either *p*H 6.5 or 8.5 led to an increasing proportion of (C) and of (B), and, to a decreasing proportion of (A). Since (C) constitutes by far the most soluble of the three fractions, it is possible to isolate pure (A) at *p*H 8.5 and at 0.015% initial concentration. Component (B) is more soluble than (A), and has seemingly never been isolated in crystalline form without some contamination by (A). Previous oxidative studies were conducted at 1.2% concentration, and at this level and at *p*H 8.5, preparations were obtained rich in (B) and with varying proportions present of (A).[4] At the highest concentration studied, namely 3%, and at *p*H 8.5, recrystallization of the product from water yielded a small quantity of a flocculent material which was shown by paper chromatography (phenol) to consist mainly of (B) with some (C) and only a trace of (A) present. At 3% concentration and at *p*H 6.5, little or no (A) is formed.

Effect of *p*H.—Solutions of L-cysteinyl-L-cysteine at 0.15% concentration in water were brought to various *p*H values in the range of 4.5 to 10.7 and aerated to disappearance of the nitroprusside reaction. The solutions were condensed *in vacuo* and the material which separated was recrystallized from water. The compounds were subjected to polarimetric and chromatographic analysis, the latter in phenol, and the data are given in Table II. At every *p*H studied, the crystalline product contained (A) in appreciable amounts but only as a result of the oxidation carried out in the *p*H range 7.0 to 9.6 was (B) also present. On either side of this range relatively little (B) must have been formed, and recrystallization of the product from water evidently removed both (B) and any adherent (C) allowing thereby the isolation of pure (A). In confirmation of the earlier results,[4] the $[\alpha]_D$ of pure (A) with R_f = 0.49 (phenol) was close to −30°, and with increasing amounts of (B) present in the preparation, the magnitude of the negative rotation increased. Studies of the purest preparation of (B) which have so far been obtained, *i.e.*, those with the least contamination by (A), suggest that the $[\alpha]_D$ value of (B) is not far from −60°.[3,4] A preparation obtained by the oxidation of L-cysteinyl-L-cysteine at *p*H 8.5 and at 3% concentration (*cf.* Table I) was composed mainly of (B) with some (C) and a trace of (A) present, and possessed an $[\alpha]^{25}D$ −99.8° (*c* 1, 1 *N* HCl). Two recrystallizations from water served to reduce but not eliminate the presence of (C), and resulted in a diminution of $[\alpha]^{25}D$ to −86°. Each crystallization was accompanied by great loss of material, and further purification could not be obtained because of paucity of material. Crystallization is not always a reproducible process, and the proportion of components noted in a preparation may depend upon several adventitious factors. Thus, the final preparation obtained at *p*H 8.0 (Table II) in which the proportion of (B) appeared to be unusually low, may well have been due to conditions coincidental to the particular crystallization steps involved in this preparation. In any event, frequent attempts by fractional crystallization to separate the (A) and (B) components in a mixture have generally resulted in the isolation of mixtures increasingly richer in (A), the less soluble component.

Rate of Oxidation.—At 0.15% concentration and a nearly constant rate of aeration, the time required to oxidize equal amounts of cysteinylcysteine was about 3 days at *p*H 4.5 and 5.0, about 4 hours at *p*H 6.5 to 8.5, and about 6 to 12 hours at *p*H 9.6 and 10.7. When porphyrindin was used at *p*H 7.0 to oxidize the compounds quantitatively

(6) We wish to express our appreciation of the help and many pertinent and valuable suggestions given us by Drs. H. Sober and E. Peterson in the course of the chromatographic studies herein reported.

(7) Composed of 160 parts of methyl ethyl ketone, 160 parts of *t*-butyl alcohol, 39 parts of water, and 1 part of formic acid.

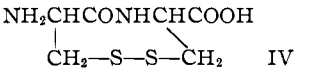
(8) Composed of phenol saturated with 10% sodium citrate solution, and run in the presence of 0.3% ammonia and of sodium cyanide in phenol.

and in a matter of seconds,[9] the same distribution of oxidized forms was obtained as with aeration over a period of hours (*cf.* Tables I and II).

Molecular Weight Determinations.—A method of determining the molecular weight of relatively small compounds by sedimentation has been developed by Klainer and Kegeles.[10]

The method has been used in a determination of the molecular weight of raffinose (M = 504), the experimental error being about 3%.[10] In order to ensure the validity of the procedure for dipolar ions of this molecular size, a determination was carried out on a simple peptide of known molecular weight, namely, glycyl-L-leucine. The results on this compound and on the peptides of L-cystine are given in Table III.

The probable experimental error was for glycyl-L-leucine about 3%, for (A) about 6%, and for (A + B) about 3%. The data in Table III for glycylleucine show that the method is readily applicable to the determination of the molecular weight of small peptides. The results on the sample of pure (A) show that this compound, with $[\alpha]^{25}D$ −29°, and prepared by oxidation of L-cysteinyl-L-cysteine in 0.15% aqueous solution and *p*H 6.5, is a monomer, and represented by the formulation (IV)



IV

To this compound, the designation of "cyclo-L-cystinyl" has been given.

The molecular weight of the L-cystine peptide (A + B) which has an $[\alpha]^{25}D$ −47°, and which was formed by oxidation of L-cysteinyl-L-cysteine in 0.15% aqueous solution at *p*H 8.5, was found to be 363. This value is a weight average of the molecular weights of (A) and (B), which were noted by paper chromatography to be the only ninhydrin-reactive components present in the peptide preparation. Inasmuch as (A) is a monomer with a molecular weight of 222, and (B), from the earlier data is a dimer[3] (II or III) with a molecular weight of 444, it is possible to calculate from the molecular weight of the mixture, namely 363, the relative proportion of (A) to (B) according to the equation

$$M = \frac{nm_1^2 + (1-n)m_2^2}{nm_1 + (1-n)m_2}$$

The calculation leads to 53 parts of (A) to 47 parts of (B) in the preparation studied.

If the assumption is made that no fractionation of the components was made in the preparation of the (A + B) solution for the sedimentation studies, *i.e.*, that this solution is the same as that employed to determine the value of $[\alpha]D$, it is possible to calculate approximately the $[\alpha]D$ value for the (B) component from the observed $[\alpha]^{25}D$ values of −47° for the preparation, and of −29° for the (A) component, and from the relative amounts of (A) and of (B) in the preparation. The results lead to a value of −68° for the $[\alpha]^{25}D$ of the (B) peptide which may be compared to that of −59° for that preparation of (B) which by chromatographic evidence contained the least amount of (A) as a contaminant,[4] or to that of −60° for the preparation of L-cystinyl-L-cystine obtained several years ago.[3,11]

TABLE II

EFFECT OF *p*H ON THE NATURE OF THE PEPTIDES OF L-CYSTINE FORMED BY OXIDATION OF L-CYSTEINYL-L-CYSTEINE[a]

*p*H of oxidation	Yield of products, %	$[\alpha]^{25}D$[b]	Intensity of ninhydrin-reactive spots using phenol solvent for[c] (A) R_f = 0.49	(B) R_f = 0.18	Found for[d] C	H	N	S
4.5	25	−29.5	Strong	Absent	..	..	..	..
5.0	32	−29.1	Strong	Absent	32.8	4.7	12.7	..
6.5	72	−29.3	Strong	Absent	32.6	4.7	12.6	28.4
7.0	70	−33.0	Strong	Faint	32.4	4.8	12.6	28.4
7.5	68	−47.5	Strong	Strong	32.4	4.8	12.6	28.5
8.0	63	−36.2	Strong	Weak	32.6	4.9	12.7	28.4
8.5	70	−47.2	Strong	Strong	32.1	4.8	12.7	28 8
9 6	61	−30.5	Strong	Trace	32.7	4.6	12.7	28.6
10.7	55	−28.8	Strong	Absent	32.4	4.8	12.7	28.7

[a] All solutions of L-cysteinyl-L-cysteine initially at 0.15% in water. [b] Concentration = 1, in 1 *N* HCl. [c] No trace of L-cystine present in (A) or (B) as determined by paper chromatography using both ketone mix and phenol–citrate buffer as solvents. [d] Calcd. for $(C_6H_{10}O_3N_2S_2)_x$: C, 32.4; H, 4.5; N, 12.6; S, 28.8. There was an insufficient amount of the sample prepared at *p*H 4.5 for analysis. All compounds were dried *in vacuo* to constant weight.

TABLE III

MOLECULAR WEIGHT OF PEPTIDES BY SEDIMENTATION

Compound	$[\alpha]^{25}D$[a]	M Found	M Calcd.
Glycyl-L-leucine	...	185, 185, 193	188
L-Cystine peptide (A)	−29°	231	222 (for monomer)
L-Cystine peptide (A + B)	−47°	363	.....

[a] *c* 1, in 1 *N* HCl.

Dinitrophenyl Derivatives.—Application of the dinitrophenyl technique afforded further evidence, compatible with the foregoing, for the structure of these compounds. The monomeric cyclic peptide (A) with $[\alpha]^{25}D$ −29° formed by oxidation of L-cysteinyl-L-cysteine at *p*H 6.5, and a mixture of (A) and (B) possessing an $[\alpha]^{25}D$ −47° and formed by oxidation of L-cysteinyl-L-cysteine at *p*H 8.5 were each converted to the crystalline dinitrophenyl derivatives. Both products possessed the same R_f values in *t*-amyl phthalate and could not be separated on paper.

If structure IV for (A) is correct, the hydrolysis of DNP-(A) should yield only mono-DNP-L-cystine. This was found to be the case, a water-

(9) J. P. Greenstein, *J. Biol. Chem.*, **125**, 501 (1938).

(10) S. M. Klainer and G. Kegeles, *J. Phys. Chem.*, **59**, 952 (1955), The authors are greatly indebted to Dr. Kegeles for informing them of this method prior to publication, and permitting R. W. to carry out measurements on the compounds in his laboratory at Clark University.

(11) With the determination that (A) is a monomer, the analytical data previously interpreted on the basis that this compound was a dimer,[4] now indicate that this compound crystallizes from water as a monohydrate, and forms a monohydrochloride salt which crystallizes with one molecule of water of crystallization. The nearly pure dimeric (B) peptide crystallizes with two molecules of water of crystallization and forms a dihydrochloride salt which crystallizes with four molecules of water of crystallization.[3,4] The ultraviolet and infrared spectra of (A) and of (B) are practically indistinguishable, *cf.* M. C. Otey and J. P. Greenstein, *Archiv. Biochem. Biophys.*, **53**, 501 (1954), as are also the titration curves of the two peptides, although the titration constants of the monomer should now be expressed in terms of two constants, namely, $pK_1' = 2.51$, and $pK_2' = 7.11$.

soluble, yellow compound giving a positive ninhydrin reaction being obtained which was indistinguishable on paper chromatography from a known sample of mono-DNP-L-cystine. Chromatographic analysis of both ether and aqueous extracts of the hydrolysate revealed no trace of either cystine or di-DNP-cystine.

Hydrolysis of the DNP derivative of the (A) plus (B) mixture yielded evidence for the presence of mono-DNP-cystine and, in addition, clear evidence for the presence of considerable quantities of cystine and of di-DNP-cystine. Both cystine and di-DNP-cystine would be expected to arise by hydrolysis of the DNP derivative of the parallel structure II, designated throughout as (B). Like IV, the DNP derivative of the antiparallel dimeric structure III should yield mono-DNP-cystine on acid hydrolysis. Consequently, the occurrence of this antiparallel isomer in a mixture in which cyclocystinyl, *i.e.*, (A), is present, cannot be determined by this technique. It must be recalled, however, that in such mixtures only two ninhydrin-reactive spots appear on the paper chromatograms in several solvents, and one of these, namely, the faster-moving, is due to (A). If the anti-parallel isomer III should form a third component in the mixture, it must possess a chromatographic behavior indistinguishable from that of isomer IV or isomer II. In any event, the tentative designation of L-cystinyl-L-cystine (B) largely if not wholly as the dimeric structure II is compatible with all of the evidence so far accumulated.

Experimental

Preparation of Peptides.—The peptides of L-cystine were prepared substantially as described in part I of this series.[4] Both benzyl groups of S-benzyl-L-cysteinyl-L-cysteine were removed by reduction using sodium in liquid ammonia, and the resulting L-cysteinyl-L-cysteine purified as the insoluble mercury mercaptide. Decomposition of the mercury derivative was effected with hydrogen sulfide, and, after filtration from mercury sulfide, the solution was freed from excess hydrogen sulfide by evaporation *in vacuo*. The *p*H of the solution at this point was about 1.5. Adjustment to the *p*H desired was carried out by addition of baryta. After filtration of the barium sulfate through a pad of Hyflo Super-Cel, the clear filtrate was diluted with water or concentrated by evaporation to the desired volume and the *p*H again checked. A stream of air previously passed through a water bubbler was introduced into the cysteinylcysteine solution. Solutions at *p*H 7.0 and over were a pale purple color, probably due to the presence of traces of heavy metal. During aeration the purple color faded and was eventually replaced by a pale yellow-green color about 30 minutes before the solution no longer gave a positive color reaction with sodium nitroprusside or ferric chloride. The period of time required for complete oxidation depended in large degree on the *p*H of the solution. Thus, at 0.15% concentration, the period of time with a nearly constant rate of aeration was about 3 days at *p*H 4.5 and 5.0, about 4 hours at *p*H 6.5 to 8.5, and about 6 to 12 hours at *p*H 9.6 and 10.7. Each solution contained approximately 0.5 g. of cysteinylcysteine. The periods of time given are admittedly highly approximate, but to see whether, by drastically reducing the time required for oxidation, a different spectrum of oxidized products could be obtained, the use of the high oxidation potential dye porphyrindin was resorted to.[9] The same solution of cysteinylcysteine at *p*H 7.0 which required about 4 hours of aeration to effect complete oxidation was treated with an aqueous solution of porphyrindin until the blue color of the dye was maintained for several seconds. The entire titration required less than a minute, and at the end the nitroprusside reaction was negative. The oxidized products obtained were identical with those obtained by the aeration procedure (Table II).

Differing concentrations of L-cysteinyl-L-cysteine at *p*H 6.5 and at *p*H 8.5 were prepared and, after oxidation was complete, aliquots of the solutions were chromatographed in phenol on Whatman No. 1 and Schleicher and Schull No. 598 paper. The results are given in Table I of the text.

When isolation of the oxidized peptides was desired, the solutions were brought to *p*H 5 by addition of sulfuric acid. In the case of oxidations carried out at 3% concentration, it was necessary to dilute the solutions to six times the volume prior to adjustment of *p*H to 5 in order to prevent the crystallization of peptide at this stage. The precipitate of barium sulfate was removed by filtration, the filtrate evaporated *in vacuo* and at low temperature to a small bulk, and the crystals filtered off. Recrystallization was generally effected by dissolving the solid in a large volume of warm water, filtering from a trace of insoluble impurity, and re-evaporating to a small bulk. The analytical results on the various preparations have been described in Table II.

Molecular Weight Determinations.—The present measurements were carried out in acetate buffer solution at *p*H 4.5. The concentration of the solution was measured in a 2° synthetic boundary cell, the boundary being formed at about 500 r.p.m. and the picture taken at 2,000 r.p.m. Sedimentation runs were carried out on the peptide solutions using a 4° standard cell, pictures being taken as equilibrium was approached beginning 3 minutes after full speed (60,000 r.p.m.) was established and continuing at 8-minute intervals thereafter. The equation developed by Klainer and Kegeles to ascertain the molecular weight is

$$M = \frac{RT\left(\frac{dc}{dx}\right)_{x_0}}{(1 - V\rho)c_{x_0}\,4\pi^2\nu^2}$$

wherein $(dc/dx)_{x_0}$ is the height of the sedimentation curve at the meniscus, V is the specific volume of the peptide, ρ is the density of the solution, x_0 is the radius of rotation at the meniscus, ν is the frequency of rotation, and c_{x_0}, the factor for the concentration at the meniscus is defined as

$$c_{x_0} = \frac{0.05}{m}\left(\sum_{x_a}^{x_b}\frac{dc}{dx} - \frac{1}{x_0^2}\sum_{x_0}^{x}x^2\right)$$

The expression m is the magnification factor and 0.05 is the interval in cm. along the x-axis. Values of V employed were 0.61 for the peptides of cystine, and 0.74 for glycyl-L-leucine.

Dinitrophenyl Derivatives.—These were prepared by dissolving the cystine peptides (1 g.) in 50% aqueous alcohol containing sodium bicarbonate (0.9 g.). Dinitrofluorobenzene (0.6 ml.) was added and the mixture shaken for 2 hours. The alcohol was removed by evaporation and the solution made strongly acid with 6 *N* HCl. The yellow derivative was filtered at the pump, sucked dry, washed repeatedly with ether, and finally crystallized from aqueous isopropyl alcohol.

Calcd. for $C_{12}H_{12}O_7N_4S_2$: C, 37.1; H, 3.1; N, 14.4; S, 16.5. Found (for DNP-(A)): C, 37.3; H, 3.5; N, 14.7; S, 16.4. Found (for DNP-(A + B)): C, 37.3; H, 3.4; N, 14.3. The two DNP-peptides possessed identical values of R_f, namely, 0.72 in *t*-amyl phthalate with S and S No. 598 paper. The use of other solvents, such as toluene–pyridine, phenol–HCN–NH_3–citrate, or M phosphate gave various R_f values which were identical for the DNP (A) and (A + B) preparations.

Hydrolysis of the DNP-peptides was effected by dissolving the derivative (1 g.) in 30% ethanolic 2 *N* HCl (500 ml.) and refluxing the solution for 3 hours. The solution was evaporated *in vacuo* to dryness and the residue shaken with dilute HCl (100 ml.) and with ether (100 ml.). The layers were separated, and each was evaporated to dryness and the residues chromatographed. In the case of the derivative prepared from the (A + B) mixture, both ether and aqueous layers possessed a distinctly yellow color, but in the case of the (A) derivative alone the yellow color was almost entirely found in the aqueous layer. The reference compounds consisted of L-cystine, di-DNP-L-cystine, and mono-DNP-L-cystine, the last-mentioned being prepared by the method of Bettelheim.[12]

(12) F. R. Bettelheim, *J. Biol. Chem.*, **212**, 235 (1955). The melting point of mono-DNP-L-cystine reported in this reference was 187°. Like Ryle and Sanger[1] we have noted a melting point of 179° for this compound.

The hydrolysate of DNP-(A) yielded mono-DNP-cystine (R_f = 0.62 in phenol–citrate–cyanide–ammonia mixture), and in addition two yellow spots, one ninhydrin-reactive with R_f = 0.85, and the other ninhydrin-negative with R_f = 0.93. That the latter two spots arise by decomposition of mono-DNP-cystine during the hydrolytic procedure was shown by subjecting this derivative to the boiling acid solvent and taking paper chromatograms at hourly intervals. The two spots gradually made their appearance with increasing intensity as the boiling proceeded. A similar experiment with DNP-(A) revealed the same phenomenon. No trace of cystine (R_f = 0.22) or of di-DNP-cystine (R_f = 0.81) was ever detected among the hydrolytic products under these conditions. However, in a refluxing solvent composed of 3 *N* HCl in 25% formic acid, DNP-(A) yielded, in addition to mono-DNP-cystine, two spots on paper; one of these was yellow and ninhydrin-insensitive (R_f = 0.76), and the other was colorless, ninhydrin-reactive, and identified unmistakably as due to cystine.

The hydrolysate of DNP-(A + B) yielded clear evidence of cystine in the aqueous extract (R_f = 0.22) and di-DNP-cystine (R_f = 0.81) in the ether extract. In addition there was clear evidence for the presence of mono-DNP-cystine as well as for small amounts of its decomposition products. In all cases mono-DNP-cystine was recognized by its solubility in aqueous media and by its chromatographic behavior, moving as a yellow spot which on development with ninhydrin became purple-brown in color.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN CO.]

Metabolite Inhibitors. I. 6,7-Dimethyl-9-formylmethylisoalloxazine, 6,7-Dimethyl-9-(2′-hydroxyethyl)-isoalloxazine and Derivatives[1]

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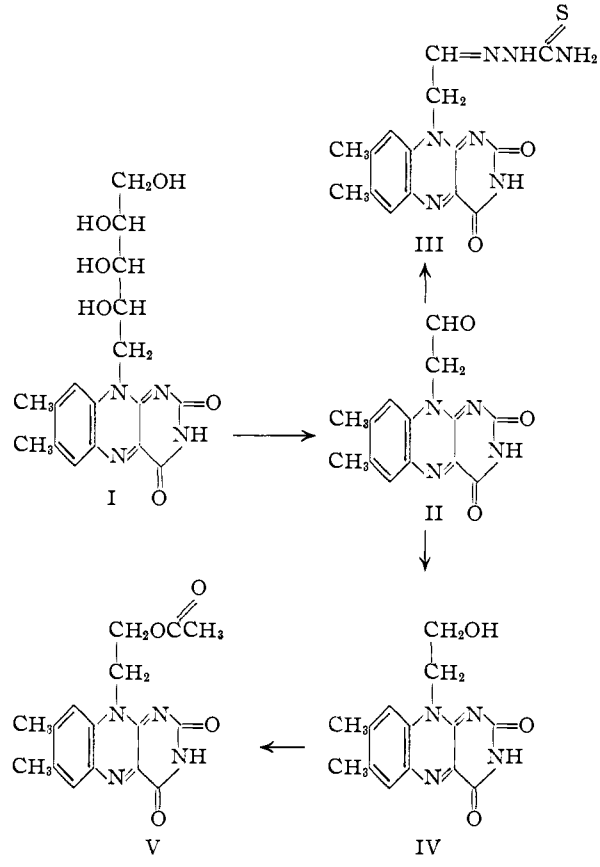
A series of biologically active riboflavin antagonists—6,7-dimethyl-9-formylmethylisoalloxazine (II), 6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine (IV) and 6,7-dimethyl-9-(2′-acetoxyethyl)-isoalloxazine (V)—has been prepared. Other derivatives of II and IV also have been made. Physical properties are presented. The use of ultraviolet absorption spectra on the quantitative level and saponification numbers is demonstrated.

Introduction

The development of a potent riboflavin antagonist is of interest for fundamental studies in metabolism as well as for investigation of possible chemotherapeutic activity. That analogs of riboflavin inhibit flavoenzymes or influence riboflavin metabolism in a variety of biological systems provided a reason to prepare a number of isoalloxazines for evaluation of their therapeutic activity against experimental cancer and as antiparasitic and antimicrobial agents. There are only a few riboflavin antagonists of the isoalloxazine structure effective in either animal or both animal and microorganism systems.[2–5] The structural criteria for biological activity appear to be vicinal alkyl groups in the 5,6- or 6,7-positions and sugar residues of pentose or hexose origin in the 9-position. This paper constitutes the first in a series which will discuss the chemistry and biochemistry of a group of isoalloxazine compounds with a 2-carbon residue in position 9 having interesting biological activity, possible therapeutic value and reversible riboflavin antagonism in animals as well as in systems of microörganisms. Specifically, this report covers 6,7-dimethyl-9-formylmethylisoalloxazine (II), 6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine (IV) and certain of their derivatives.

During the period in which the structure of riboflavin was being elucidated, Kuhn[6] reported that the oxidation of riboflavin with an excess of lead tetraacetate in glacial acetic acid gave 78% of the theoretical amount of formaldehyde expected from the vitamin B_2 compound if it had only one primary aliphatic alcohol group in the tetrahydroxyamyl sidechain. However, no other identifiable compounds were isolated from this reaction which was carried out strictly for analytical purposes.



(1) Presented at the 126th Meeting of the American Chemical Society, New York, N. Y., September, 1954.

(2) G. A. Emerson and M. Tishler, *Proc. Soc. Exptl. Biol. Med.*, **55**, 184 (1944).

(3) H. v. Euler and P. Karrer, *Helv. Chim. Acta*, **29**, 353 (1946).

(4) G. A. Emerson, E. Wurtz and O. H. Johnson, *J. Biol. Chem.*, **160**, 165 (1945).

(5) J. P. Lambooy and H. V. Aposhian, *J. Nutrition*, **47**, 539 (1952).

(6) R. Kuhn, H. Rudy and T. Wagner-Jauregg, *Ber.*, **66B**, 1950 (1933).