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3-Carboxy-2,5-piperazinedione and Derivatives

BY H. E. ZAUGG, M. FREIFELDER, H. J. GLENN, B. W. HORROM, G. R. STONE AND M. R. VERNSTEN

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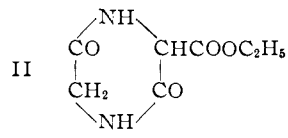
Hydrogenolysis of diethyl carbobenzyloxyglycylaminomalonate (I) gave the expected 3-carbethoxy-2,5-piperazinedione (II). Derivatives of II substituted in the 1-, 3- or 6-positions likewise originated from suitably substituted derivatives of I. Hydrolysis of II led to the title compound. Other reactions of the ester function of II and its active α-hydrogen atom were also studied.

Introduction.—Since the great majority of known substituted 2,5-piperazinediones are derived from combinations of the natural amino acids, examples of this class, in which the ring is directly substituted by a functional group, are quite rare. Preliminary tests indicated that one such compound, 3-carbethoxy-2,5-piperazinedione (II), was protective against the MM strain of poliomyelitis virus in mice. However, by the time the synthetic expansion based on this evidence was nearly completed, it became apparent that these precursory therapeutic results were misleading. Since examples of this particular type do not appear to have been reported previously, the preparation, proof of structure and properties of these compounds provides the subject matter of this communication.

Preparation and Reactions.—The most generally useful method for the preparation of compounds of type II was provided by the hydrogenolysis of carbobenzyloxyaminoacylaminomalonic esters I, accompanied in all cases by spontaneous ring closure (method C). Indeed, in two instances appreciable cleavage and cyclization of the carbobenzyloxy derivatives even in the absence of reducing conditions was noted (method D).

$$\underset{\text{I}}{C_6H_5CH_2OCONHCH_2CONHCH(COOC_2H_5)_2} \xrightarrow{H_2\text{–Pd}}$$



II

Intermediates of type I were secured by acylation of the aminomalonic ester by the appropriate carbobenzyloxyamino acid chloride according to Schneider's[1] method for preparing I (methods A and B). In addition to structural variation of the aminoacyl part, substitution of the α-position of the aminomalonic ester portion of I was achieved, usually by alkylation of the sodium derivative of I with a suitable alkyl halide (method N). Also direct alkylation of the sodium derivative of aminomalonic ester with ethyl iodide[2] followed by acylation with carbobenzyloxyglycyl chloride led to the same product obtained by the ethylation of the sodium derivative of I. In addition base-catalyzed cyanoethylation of I with acrylonitrile (method O) led to the α-(β-cyanoethyl)-derivative of I.

Acylation of *dimethyl* aminomalonate with carbobenzyloxyglycyl chloride led to the dimethyl ester analogous to I, and subsequent hydrogenolysis furnished the methyl ester analogous to II.

All of the intermediates of type I prepared in this work are listed in Table I.

TABLE I

SUBSTITUTED CARBOBENZYLOXYAMINOACETYLAMINOMALONIC ESTERS

$C_6H_5CH_2OCON(R_1)CH(R_2)CONHC(R_3)(COOC_2H_5)_2$

Compound			M.p., °C.	Method	Yield, %
R_1	R_2	R_3			
H	H	H	96–97[a]	B	79
H	H	H	135–138[b]	A	37
CH_3	H	H	75.5–77[c]	B	66
H	CH_3[d]	H	93–94	A	29
H	$C_6H_5CH_2$[e]	H	109–110	A	90
H	C_6H_5[f]	H	110–112	A	43
H	*i*-C_4H_9[g]	H	Oil	A	32
H	H	CH_3	Oil	N	79
H	H	C_2H_5	Oil	N	82
				B[h]	72[i]
H	H	*n*-C_4H_9	Oil	N	81
H	H	*n*-$C_{12}H_{25}$	Oil	N	60[j]
H	H	$C_6H_5CH_2$	Oil	N	86
H	H	$NCCH_2CH_2$	Oil	O	86

[a] Previously prepared by Schneider.[1] [b] Dimethyl ester (all other compounds listed are diethyl esters, as indicated). *Anal.* Calcd. for $C_{15}H_{18}N_2O_7$: C, 53.25; H, 5.36. Found: C, 53.14; H, 5.18. [c] *Anal.* Calcd. for $C_{18}H_{24}N_2O_7$: C, 56.83; H, 6.36. Found: C, 57.07; H, 6.14. [d] *dl*-Alanyl. *Anal.* Calcd. for $C_{18}H_{24}N_2O_7$: C, 56.83; H, 6.36; N, 7.37. Found: C, 56.62; H, 6.26; N, 7.47. [e] *dl*-Phenylalanyl. *Anal.* Calcd. for $C_{24}H_{28}N_2O_7$: C, 63.14; H, 6.18; N, 6.14. Found: C, 62.57; H, 6.12; N, 6.25. [f] *dl*-Phenylglycyl. *Anal.* Calcd. for $C_{23}H_{26}N_2O_7$: C, 62.43; H, 5.92; N, 6.33. Found: C, 62.49; H, 5.77; N, 6.50. [g] *l*-Leucyl. [h] The starting material, diethyl ethylaminomalonate, b.p. 68–70° (0.8 mm.), $n^{25}D$ 1.4301, was prepared in 50% yield (*Anal.* Calcd. for $C_9H_{17}NO_4$: N, 6.89. Found: N, 6.81) by the method of Locquin and Cerchez.[2] [i] Analytical sample was prepared by filtering the oil through a fine sintered glass funnel followed by drying at 78° under a pressure of 1 mm. *Anal.* Calcd. for $C_{19}H_{26}N_2O_7$: C, 57.86; H, 6.64. Found: C, 57.79; H, 6.70. [j] A 14% yield of the corresponding cyclized diketopiperazine (Table IV) was obtained also.

Derivatives of II substituted in the 1- or 6-position and prepared by hydrogenolysis of the corresponding derivatives of I are listed in Table III. Similarly, 3-substituted derivatives of II are recorded in Table IV. Data, concerning compound II itself, and a number of 2,5-piperazinediones derived from II by reaction at the functional group, are summarized in Table II.

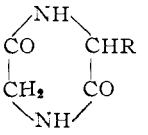
The chemical properties of 3-carbethoxy-2,5-piperazinedione (II) were studied in some detail. Rapid ester-interchange occurred under conditions catalyzed either by sodium alkoxide or by a tertiary aliphatic amine. The action of methanol–trimethylamine on II produced the corresponding

(1) F. Schneider, *Biochem. Z.*, **291**, 328 (1937).

(2) R. Locquin and V. Cerchez, *Bull. soc. chim.*, [4], **47**, 1377, 1381 1930).

TABLE II

3-SUBSTITUTED 2,5-PIPERAZINEDIONES

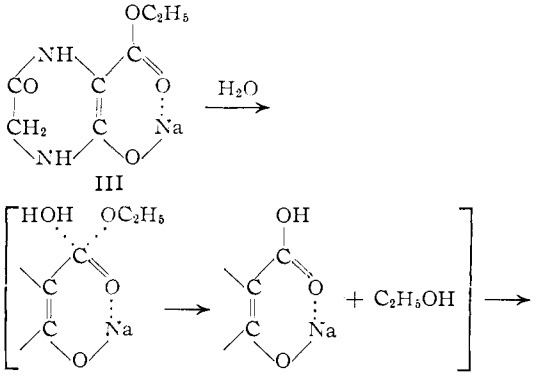


Compound R	M.p., °C.	Method	Yield, %	Formula	Analyses, % Calcd. C	Calcd. H	Found C	Found H
$COOC_2H_5$	170–172	C	90–97	$C_7H_{10}N_2O_4$[d]	45.16	5.41	45.36	5.43
$COOCH_3$	207.5–208.5	C	22	$C_6H_8N_2O_4$	41.86	4.68	42.03	4.74
	207–208	J	26	$C_6H_8N_2O_4$	41.86	4.68	41.96	4.48
	203–204.5	H	42	$C_6H_8N_2O_4$				
$COOCH(CH_3)_2$	207–210	J	12	$C_8H_{12}N_2O_4$[e]	47.99	6.04	46.83	5.69
COOH	306–308[a]	E	55	$C_5H_6N_2O_4$	37.98	3.83	37.77	3.89
	300–305[a]	F	52	$C_5H_6N_2O_4$	37.98	3.83	38.46	3.96
	305–306[a]	G	53	$C_5H_6N_2O_4$	37.98	3.83	37.77	3.94
$CONH_2$	262–264[a]	L	48	$C_5H_7N_3O_3$	38.22	4.49	38.38	4.79
	262–264[a]	K	20	$C_5H_7N_3O_3$	38.22	4.49	38.37	4.53
$CONHCH_3$	273–275[a]	L	72	$C_6H_9N_3O_3$	42.10	5.30	42.16	5.30
$CONHC_2H_5$	255–256[a]	L	66	$C_7H_{11}N_3O_3$[f]	45.40	5.99	45.32	5.70
$CONC_5H_{10}$[b]	278–280[a]	L	17	$C_{10}H_{15}N_3O_3$	53.32	6.71	53.30	6.81
$CONHCH_2CH_2N(CH_3)_2$	234–236[a]	L	75	$C_9H_{16}N_4O_3$	47.36	7.07	47.54	7.09
$CONHCH_2CH_2N(C_2H_5)_2$	237–240[a]	L	51	$C_{11}H_{20}N_4O_3$	51.55	7.87	51.69	7.83
$CH_2CH_2COOC_2H_5$	179–180	C[c]	63	$C_9H_{14}N_2O_4$[g]				
$CONHCH_2COOH$	268–270[a]	M	60	$C_7H_9N_3O_5$[h]	39.07	4.22	38.91	4.11
$CONHCH(CH_3)COOH$	240–241[a]	M	21	$C_8H_{11}N_3O_5$	41.92	4.84	41.98	4.59
$CONHCH_2COOC_2H_5$	235–237[a]	L	18	$C_9H_{13}N_3O_5$[i]	44.44	5.39	44.29	5.23

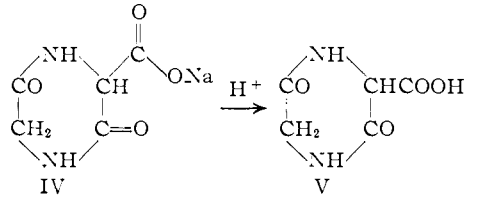
[a] With decomposition after darkening. [b] Piperidide. [c] By hydrogenolysis of diethyl carbobenzyloxyglycyl-*l*-glutamate prepared according to Prelog and Wieland, *Helv. Chim. Acta*, **29**, 1129 (1946). [d] *Anal.* Calcd.: N, 15.05. Found: N, 14.85. [e] *Anal.* Calcd.: N, 13.99. Found: N, 14.61. [f] *Anal.* Calcd.: N, 22.69. Found: N, 22.25. [g] *Anal.* Calcd.: N, 13.08. Found: N, 12.95. [h] *Anal.* Calcd.: N, 19.53. Found: N, 19.69. [i] *Anal.* Calcd.: N, 17.28. Found: N, 17.27.

methyl ester identical with the product obtained by hydrogenolysis of the methyl ester analog of I.

3-Carbethoxy-2,5-piperazinedione (II) could be made to undergo hydrolysis in a curious manner. Treatment of II with sodium ethoxide in dry ethanol gave an insoluble sodium derivative which dissolved rapidly and exothermically in a small amount of water. This solution then quickly precipitated a second sodium derivative which could be dissolved by the addition of more water. Acidification of this solution gave the free acid, 3-carboxy-2,5-piperazinedione (V). It is tempting to account for this ready conversion of sodio derivative to sodium salt by postulating that the former exists mainly in the chelate form III, which promotes nucleophilic attack by water (or HO^- ion) at the carbonyl carbon with consequent elimination of ethanol (or $C_2H_5O^-$ ion). Suitable rearrangement of a proton finally gives the normal sodium salt IV.



III



IV V

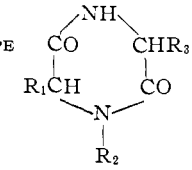
Credibility of this mechanism is enhanced by Brändström's[3] recent assertion of the importance of alkali metal chelates in the alkylation of β-dicarbonyl compounds. Similarly, when the ester II was treated with anhydrous guanidine base in an attempt to prepare the corresponding guanide (an open chain analog of dihydroxanthopterin), the only product obtained after crystallization from *aqueous* ethanol was the guanidinium salt of V. Indeed, the well-known ability of the guanidinium ion to distribute an electronic charge would be expected to promote chelate resonance interaction with the adjacent carbonyl oxygen. To be sure, hydrolysis in this manner might give the appearance of proceeding by a mechanism involving alkyl–oxygen fission.[4] However, the observed normal behavior under conditions of ester-interchange is inconsistent with abnormal reactivity of the alkyl–oxygen bond. Furthermore, the course of the reaction of the ester II with amines is compatible with the mechanism proposed above. No chelate ammonium derivative is possible.[3] Therefore, the ammonium ion could not take the part of the sodium or guanidinium ion in this mechanism. As was ob-

(3) A. Brändström, *Acta Chem. Scand.*, **7**, 223 (1953).

(4) Compare W. Cohen and A. Corwin, THIS JOURNAL, **75**, 5880 (1953).

TABLE III

2,5-PIPERAZINEDIONES OF TYPE

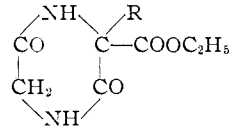


Compound							Analyses, %					
							Calcd.			Found		
R_1	R_2	R_3	M.p., °C.	Method	Yield, %	Formula	C	H	N	C	H	N
H	CH_3	$COOC_2H_5$	93.5–94	C	76	$C_8H_{12}N_2O_4$			13.99			14.05
H	CH_3	$CONHCH_3$	235–237	L[a]	93	$C_7H_{11}N_3O_3$	45.40	5.99		45.36	5.73	
CH_3	H	$COOC_2H_5$	169–170	C	86	$C_8H_{12}N_2O_4$	47.99	6.04	13.99	48.21	5.90	14.05
$C_6H_5CH_2$	H	$COOC_2H_5$	202–203	C	72	$C_{14}H_{16}N_2O_4$	60.86	5.84	10.14	60.75	5.75	10.00
$C_6H_5CH_2$	H	$CONH_2$	247–248[b]	L[c]	45	$C_{12}H_{13}N_3O_3$	58.29	5.30		58.34	5.09	
C_6H_5	H	$COOC_2H_5$	235–237	C	48	$C_{13}H_{14}N_2O_4$	59.53	5.38	10.68	59.58	5.33	10.87
i-C_4H_9[d]	H	$COOC_2H_5$	171–173	C	10	$C_{11}H_{18}N_2O_4$			11.56			11.58
$(CH_3)_2$[e]	H	$COOC_2H_5$	161–162	D	11	$C_9H_{14}N_2O_4$	50.46	6.59	13.08	50.57	6.65	13.03

[a] Aminolysis was carried out in ether solution. [b] With decomposition. [c] Ammonolysis was carried out in methanol. *l*-Leucyl, $[\alpha]^{25}D$ −27.0° (*c* 1, 95% ethanol). [e] 6,6-Dimethyl.

TABLE IV

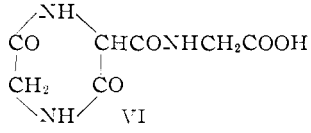
3-SUBSTITUTED-3-CARBETHOXY-2,5-PIPERAZINEDIONES



Compound, R	M.p., °C.	Method	Yield, %	Formula	Analyses, % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
CH_3	184–185	C	51	$C_8H_{12}N_2O_4$	47.99	6.04	13.99	48.40	5.99	13.93
C_2H_5	166.5–168	C[a]	78	$C_9H_{14}N_2O_4$	50.46	6.59	13.08	50.23	6.81	13.16
	168–169	C[b]	7	$C_9H_{14}N_2O_4$	50.46	6.59		50.58	6.54	
n-C_4H_9	175–175.5	C	35	$C_{11}H_{18}N_2O_4$	54.53	7.49	11.56	54.82	7.26	11.75
n-$C_{12}H_{25}$	147–148.5	C	21[c]	$C_{19}H_{34}N_2O_4$	64.37	9.67		64.52	9.62	
$C_6H_5CH_2$	234–235	C	43	$C_{14}H_{16}N_2O_4$	60.86	5.84	10.14	61.07	5.95	10.14
$NCCH_2CH_2$	205–206	C	51	$C_{10}H_{13}N_3O_4$	50.20	5.48	17.57	50.23	5.22	17.43
	207–208	P	85							
$C_2H_5OOCCH_2CH_2$	179.5–180.5	P	74	$C_{12}H_{18}N_2O_6$	50.34	6.34		50.54	6.35	
$NCCH_2CH_2$[d]	216–217		48	$C_9H_{11}N_3O_4$	48.00	4.92		48.06	4.93	

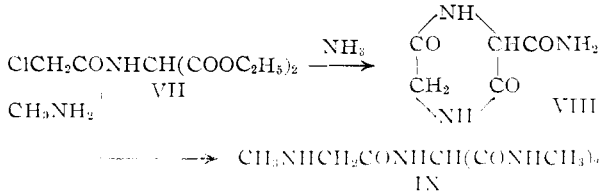
[a] Starting material (oil) was prepared by method N (see Table I). [b] Starting material (oil) was prepared by method B (see Table I). [c] An additional 14% yield of this cyclized product was formed spontaneously during preparation of the intermediate malonic ester (see Table I, footnote *j*). [d] Methyl (rather than ethyl) ester obtained as only product in attempted reaction of the ethyl ester with sodium glycinate in methanol solution (method M).

served, II behaved quite normally toward primary and secondary amines giving the expected amides, often in good yields (Table II). Even the sodium salt of glycine gave a 60% yield (after neutralization) of the unusual peptide VI.



VI

Independent synthesis of the amide VIII was accomplished by the action of ethanolic ammonia directly on chloroacetylaminomalonic ester VII. However, attempted extension of this method to methylamine gave the uncyclized diamide IX as the only isolable product.



VII VIII

IX

Although attempts to alkylate the sodio-derivative III with benzyl chloride (in refluxing toluene) failed, a replaceable α-hydrogen atom in II was demonstrated by successful Michael reactions of II with acrylonitrile and ethyl acrylate to give the corresponding 3-substituted derivatives (Table IV).

Further Structural Evidence.—Ordinarily, the multiple modes of preparation, chemical reactions and interconversions of products described above might constitute adequate evidence for the structures assigned to them. However, in 1933, Matsui[5] reported the preparation of 3-carboxy-2,5-piperazinedione (V) by the action of ammonium hydroxide on chloroacetylaminomalonic acid. His product (m.p. 193°), surprisingly, was shown to be readily attacked by trypsin. The acid V (m.p. 306–308° dec.) obtained in this work proved to be inert to the action of trypsin.

The infrared spectrum of the ester II (from which V was prepared) was found to be consistent in every way with the spectrum of 2,5-piperazinedione. Likewise the pK_a (2.5) and equivalent weight (169)

(5) J. Matsui, *J. Biochem. (Japan)*, **17**, 253 (1933).

of the acid V were compatible with the assigned structure.

Only its high degree of thermal stability seems to cast doubt on a structure (V) containing a free carboxyl group activated by a β-carbonyl group. However, analogous carboxyl substituted heterocycles such as 3-methylhydantoin-5-carboxylic acid (m.p. 130° dec.)[6] and alloxanic acid (5-hydroxyhydantoin-5-carboxylic acid, m.p. 162–163° dec.)[7] seem to show more than the expected stability. It seems likely that the stability of V is confined to its solid state in which intermolecular hydrogen bonding can contribute greatly to the crystal lattice energy. Attempts to esterify the acid V by a number of methods led neither to isolable product nor to starting material. However, by the action of diazomethane it could be converted to the methyl ester, identical with the product prepared either by ester-interchange from the ethyl ester II or by hydrogenolysis of the dimethyl acylaminomalonate. Furthermore, this methyl ester could be reconverted to the acid V by mild acid hydrolysis. Thus, the direct interconversion of these two compounds provides added evidence for the correctness of structure V.

Color Tests.—Sasaki[8] has shown that the red color, formed on heating with sodium carbonate and picric acid, is characteristic of the 2,5-piperazinedione ring. All examples of this class prepared in this work gave red colors when subjected to this test.

In addition, all 2,5-piperazinediones containing a carboxylic acid, ester or amide function directly attached to the ring, together with an α-hydrogen atom, reduced both alkaline methylene blue solution and alkaline Tollens reagent. In the absence of an α-hydrogen atom (Table IV) or when the carboxyl function was moved away from the ring (Table II, R = $-CH_2CH_2COOC_2H_5$), this reducing action disappeared.

Acknowledgments.—The authors are indebted to Dr. F. N. Minard for the enzyme studies, to Mr. William Washburn for the infrared spectra and to Mr. E. F. Shelberg for the microanalyses.

Experimental

Diethyl aminomalonate, b.p. 130–132° (26 mm.), $n^{24}D$ 1.4325, was prepared by a minor modification of the method of Snyder and Smith.[9] All known carbobenzyloxyamino acids were prepared by reported methods.[10] **N-Carbobenzyloxy-*dl*-phenylglycine** was prepared in the usual way[10a] in 83% yield, m.p. 132–133° (from CCl_4).

Anal. Calcd. for $C_{16}H_{15}NO_4$: C, 67.36; H, 5.30; N, 4.91. Found: C, 67.40; H, 5.06; N, 4.97.

Dimethyl Isonitrosomalonate.—A stirred solution of 353 g. (2.68 moles) of dimethyl malonate in 395 ml. of glacial acetic acid was treated at a temperature of 15–20° with a solution of 454 g. (6.57 moles) of sodium nitrite in 620 ml. of water. After completion of the addition, stirring at 15–20° was continued for four hours after which the mixture was allowed to stand at room temperature overnight. The mixture was then taken up in ether, washed successively with water, dilute sodium carbonate solution and more water. After drying and removing the ether by distillation, the product was distilled to give 282 g. (65%) of dimethyl isonitrosomalonate, b.p. 127–132° (3 mm.), m.p. 63–64°, $n^{25}D$ 1.4605.

Anal. Calcd. for $C_5H_7NO_5$: N, 8.69. Found: N, 8.59.

Dimethyl Aminomalonate Hydrochloride.—A solution of 161 g. (1.0 mole) of dimethyl isonitrosomalonate in 500 ml. of dry methanol was hydrogenated at room temperature and 1800 pounds pressure in the presence of 16.1 g. of 20% palladium–charcoal catalyst. Reduction was complete in less than an hour. The catalyst was removed by filtration and washed well with dry methanol. The combined filtrates were concentrated *in vacuo* below 40° to a volume of about 200 ml. and diluted with 1700 ml. of dry ether. This solution was cooled in ice and treated with excess dry hydrogen chloride until no more precipitation occurred. The product was then removed by filtration and washed with dry ether. The yield of dry, colorless, crystalline product was 156 g. (85%) which, after one recrystallization from a methanol–ether mixture, melted at 152°.

Anal. Calcd. for $C_5H_{10}ClNO_4$: N, 7.62. Found: N, 7.47.

N-Carbobenzyloxyaminoacetylaminomalonic Esters. Method A.—The procedure of Schneider[1] for the preparation of diethyl N-carbobenzyloxyglycylaminomalonate by the treatment of N-carbobenzyloxyglycyl chloride with two equivalents of diethyl aminomalonate in dry ether was followed quite closely in many cases. However, for the preparation of larger quantities of material it was found that aqueous sodium carbonate could conveniently and economically be substituted for the second equivalent of aminomalonic ester. This is exemplified in the following preparation.

Method B.—One hundred and fifty-seven grams (0.75 mole) of N-carbobenzyloxyglycine[10a] was converted to the acid chloride with PCl_5 according to the method of Schneider.[1] The solution of this acid chloride in 500 ml. of dry ether was cooled in ice and, with stirring, was treated with an ice-cold solution of 135 g. (0.77 mole) of diethyl aminomalonate in 500 ml. of dry ether, maintaining the temperature below 15° until the last portion was added; then the temperature rose to 20°. After stirring for 15 minutes in the ice-bath, a solution of 80 g. (0.755 mole) of sodium carbonate in 800 ml. of water was added at such a rate as to keep the temperature from rising above 15°. After stirring for another hour and a half in the ice-bath, the product was removed by filtration and stirred vigorously at room temperature for 30 minutes with 2500 ml. of a dilute (5–10%) aqueous solution of sodium bicarbonate. Filtration and drying gave 216 g. (79%) of diethyl N-carbobenzyloxyglycylaminomalonate (I) as a white powder, m.p. 96–97°.

Hydrogenolysis of N-Carbobenzyloxyaminoacetylaminomalonic Esters to 2,5-Piperazinediones. Method C. 3-Carbethoxy-2,5-piperazinedione (II).—A solution of 18.3 g. (0.05 mole) of diethyl carbobenzyloxyglycylaminomalonate in 200 ml. of anhydrous ethanol was hydrogenated at 30 pounds pressure and at a temperature of 70° in the presence of 3 g. of 5% palladium–charcoal catalyst. After removal of the catalyst by filtration, the filtrate was concentrated *in vacuo* to a thick paste. Addition of hexane, cooling, filtering and washing with more hexane gave the crude product, m.p. 160–165°, in 90 to 97% yield. One recrystallization from ethanol gave material, m.p. 167–168°, of sufficient purity for further work. For analysis, a sample was recrystallized from dioxane to give pure 3-carbethoxy-2,5-piperazinedione, m.p. 170–172° (Table II).

When the product was recrystallized from a benzene–ethanol mixture, shiny platelets, m.p. 174–176°, were obtained which, judging from odor and microanalytical values, even after prolonged drying, contained benzene in tightly bound form.

Attempts to prepare 3-carbethoxy-2,5-piperazinedione by reaction of diethylaminomalonate with ethyl glycinate, with glycyl chloride hydrochloride and with glycine N-carboxyanhydride all failed.

In the hydrogenolysis of other N-carbobenzyloxy derivatives, method C was varied in some cases as to temperature (25 to 70°) and initial pressure (30 to 50 pounds). Also in many instances, pretreatment of the alcoholic solu-

(6) H. Biltz, *Ber.*, **46**, 3409 (1913).

(7) H. Biltz and F. Lachmann, *J. prakt. Chem.*, **113**, 309 (1926).

(8) T. Sasaki, *Biochem. Z.*, **114**, 63 (1921).

(9) H. Snyder and C. Smith, THIS JOURNAL, **66**, 350 (1944).

(10) (a) H. Carter, R. Frank and H. Johnston, *Org. Syntheses*, **23**, 13 (1943); (b) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932); (c) M. Bergmann, L. Zervas and J. Fruton, *J. Biol. Chem.*, **115**, 593 (1936); (d) M. Bergmann, L. Zervas, J. Fruton, F. Schneider and H. Schleich, *J. Biol. Chem.*, **109**, 325 (1935); (e) D. Ben-Ishai and E. Katchalski, THIS JOURNAL, **74**, 3688 (1952).

tion with Raney nickel led to a smoother hydrogenolysis of the carbobenzyloxy group.

The corresponding 3-carbomethoxy-2,5-piperazinedione was prepared by hydrogenation of the carbobenzyloxy derivative in methanolic solution at 25° and 500 pounds pressure.

3-Carbethoxy-6,6-dimethyl-2,5-piperazinedione. Method D.—A solution in ether (200 ml.) of the acid chloride was prepared from 11.8 g. (0.05 mole) of carbobenzyloxy-α-aminoisobutyric acid[10d] in the usual manner.[10a] To this solution was added with stirring and cooling in a freezing mixture, a cold solution of 17.5 g. (0.1 mole) of diethyl aminomalonate in 100 ml. of dry ether. The temperature was kept at −5° or below. After stirring for another hour, 100 ml. of cold water was added. The ether layer was separated and dried over anhydrous sodium sulfate. Filtration and evaporation of the ether under reduced pressure gave a yellow oil which slowly deposited crystals on standing in a desiccator over phosphorus pentoxide and potassium hydroxide. Boiling with carbon tetrachloride, filtering and recrystallizing the solid thus obtained from ethyl acetate gave 1.2 g. (11%) of product, m.p. 161–162°, which proved to be 3-carbethoxy-6,6-dimethyl-2,5-piperazinedione (Table III).

3-Carboxy-2,5-piperazinedione (V). Method E. From the Sodium Derivative.—A solution of 11.6 g. (0.06 mole) of 3-carbethoxy-2,5-piperazinedione in 180 ml. of warm anhydrous ethanol was treated with a solution of 1.38 g. (0.06 g.-atom) of sodium in 35 ml. of dry ethanol. The light yellow sodium derivative precipitated at once in nearly quantitative yield. It was collected at the filter, dried, and dissolved in a minimum quantity of water. The solution warmed spontaneously and a different sodium compound gradually precipitated. More water was added to produce a clear yellow solution, showing a green fluorescence. Addition of concentrated hydrochloric acid (acid to congo red) followed by vacuum concentration to the point of crystallization, cooling in ice, and filtering gave 6.6 g. of a white powder. This was dissolved in 20 ml. of warm water, cooled in ice and treated with eight to ten drops of concentrated hydrochloric acid, whereupon 2.3 g. of a crude fraction was deposited and removed by filtration. Excess concentrated hydrochloric acid was added to the cold filtrate and 2.6 g. of 3-carboxy-2,5-piperazinedione, m.p. 306–308° (decomposing after darkening at 250–260°), was precipitated.

Anal. Calcd. for $C_4H_8N_2O_2(COOH)$: neut. equiv., 158. Found: neut. equiv., 169.

Employing the above procedure using 111.6 g. (0.6 mole) of the ethyl ester gave 51.5 g. (55%) of the carboxylic acid V.

Method F. From the Guanidine Derivative.—A solution of 2.81 g. of guanidine hydrochloride in 25 ml. of dry ethanol was combined with a solution prepared by the addition of 0.685 g. of sodium to 25 ml. of dry ethanol. The precipitated sodium chloride was removed by filtration and the filtrate was added to a solution of 4.55 g. of 3-carbethoxy 2,5-piperazinedione in 75 ml. of dry ethanol. After standing one day at room temperature, 5.04 g. of crude product was removed by filtration. Two recrystallizations from a water–ethanol mixture gave 3 g. of the guanidinium salt of 3-carboxy-2,5-piperazinedione, in the form of long colorless prisms, darkening at 180–190° and decomposing at 210–230° without melting.

Anal. Calcd. for $C_6H_{11}N_5O_4$: C, 33.18; H, 5.11. Found: C, 33.23; H, 5.07.

Treatment of this salt with concentrated hydrochloric acid by method E gave the free acid V identical with the material obtained from the sodium salt.

Method G. From the Methyl Ester.—Since the sodium derivative of the methyl ester (see method H) proved to be soluble in methanol and hence not readily isolable, direct acid hydrolysis was finally employed. To 5 ml. of ice-cold concentrated hydrochloric acid was added 1.9 g. of 3-carbomethoxy-2,5-piperazinedione and the mixture was refrigerated for three days. The precipitated product was removed by filtration in a sintered glass funnel and it was then washed successively with cold water, dry ethanol and dry ether. Drying gave 0.93 g. (53%) of analytically pure acid V possessing the properties characteristic of the material prepared by methods E and F.

3-Carbomethoxy-2,5-piperazinedione. Method H. From the Acid V.—A suspension of 0.790 g. (0.005 mole) of 3-carboxy-2,5-piperazinedione in 15 ml. of methanol was boiled for a few minutes, cooled to room temperature and treated with about two equivalents of diazomethane in ether solution. After standing overnight, the solvent was removed by distillation and the above process was repeated. Evaporating the solvent, dissolving the residue in 15 ml. of boiling methanol, filtering, seeding and cooling gave 0.362 g. (42%) of the desired methyl ester, m.p. 203–204.5° dec. When mixed with a sample of the methyl ester, m.p. 207.5–208.5° dec., prepared by method C, it melted at 203–205° dec.

Direct esterification of the acid with methanol could not be effected.

Method J. From the Ethyl Ester II.—A solution of 5.0 g. of 3-carbethoxy-2,5-piperazinedione in 100 ml. of dry methanol was treated with a solution prepared by adding 0.1 g. of sodium to 15 ml. of dry methanol. After standing for 48 hours at a temperature of 35–40°, the precipitate which formed was removed by filtration and recrystallized from methanol to give 1.2 g. of shiny leaflets, m.p. 207–208° dec. A mixture with a sample prepared by procedure C gave no depression of melting point.

The isopropyl ester prepared in a similar manner could not be obtained in analytically pure form (see Table II).

3-Carbamyl-2,5-piperazinedione (VIII). Method K. From Diethyl Chloroacetylaminomalonate (VII).—Four grams of diethyl chloroacetylaminomalonate, prepared in 94% yield by the method of Matsui,[5] was added to 200 ml. of a saturated solution of dry ammonia in absolute ethanol. The material dissolved and a solid slowly precipitated. After standing for 9 days at room temperature, the mixture was concentrated to dryness and the residue (3 g.) was crystallized from 6 ml. of water to which 8 drops of glacial acetic acid had been added. There was obtained 0.5 g. of amide in the form of shiny leaflets which decomposed rapidly at 262–264° after darkening at about 240°.

Method L. By Ammonolysis of the Ethyl Ester II.—A solution of 5 g. of 3-carbethoxy-2,5-piperazinedione in 40 ml. of dry ethanol was added to 110 ml. of a saturated solution of dry ammonia in ethanol and allowed to stand at room temperature for six hours. After refrigerating overnight, the precipitated product (3.9 g.) was removed by filtration and recrystallized by dissolving it in 50 ml. of hot water, filtering and adding 100 ml. of 95% ethanol to the filtrate. Cooling gave 2.0 g. of the amide with the same characteristics as the material prepared according to method K.

In using method L to prepare substituted amides, it was often advantageous to vary the solvent used in the aminolysis reaction. Thus for the preparation of the piperidide, the dimethylaminoethylamide and the diethylaminoethylamide, methanol was used. In making the N-methyl and N-ethylamides, dry ether, in which neither starting materials nor products are soluble, served most efficiently as a reaction medium. The N,N-diethylamide could not be obtained by any of these modifications.

Sarcosylaminomalon-N,N′-dimethylamide Hydrochloride (IX).—To a solution of 250 g. of dry methylamine in 750 g. of anhydrous ethanol was added 32 g. of diethyl chloroacetylaminomalonate.[5] After the solution was kept at room temperature for 13 days, it was clarified by filtration and concentrated to dryness *in vacuo*. The residual yellow glass was triturated with hot isopropyl alcohol and the resulting solid (17.8 g.) was removed by filtration and recrystallized from methanol to give 11.5 g. of the amide in the form of fine, filamentous needles, m.p. 229–230° dec.

Anal. Calcd. for $C_8H_{17}ClN_4O_3$: C, 38.02; H, 6.78; N, 22.17; Cl, 14.03. Found: C, 37.89; H, 6.87; N, 21.89; Cl (ionic), 13.72.

The N-(2,5-Piperazinedione-3-carbonyl)-glycine (VI). Method M.—Sodium (0.71 g., 0.031 g.-atom) was added to 40 ml. of dry methanol and after reaction was complete, 2.3 g. (0.0307 mole) of glycine was dissolved in the warm solution. After cooling to room temperature, 5.58 g. (0.03 mole) of 3-carbethoxy-2,5-piperazinedione was added with stirring. A light yellow solution formed and precipitation soon began. After stirring at room temperature for 23 hours, the sodium salt of the product (8.0 g.) was removed by filtration and dried. This was dissolved in a minimum amount of water at room temperature and acidified with concentrated hydrochloric acid. Cooling, filtering and drying gave 3.9 g. (60%), m.p. 250–254° dec. Two more recrystallizations from water gave analytically pure product (Table II).

When *dl*-alanine was substituted for glycine in the above

procedure, the corresponding peptide was obtained in only 21% yield. However, the following amino acids were tried in the above procedure, but with complete lack of success: *dl*-tryptophan, *dl*-phenylalanine, *l*(+)-glutamic acid, sarcosine and β-alanine.

Although the ethyl ester of glycine gave a peptide ester using method L, glycine amide led to no isolable product.

α-Substituted Carbobenzyloxyglycylaminomalonic Esters. Method N. By Alkylation of I. Preparation of the α-Methyl Derivative.—To a solution of 1.20 g. (0.052 mole) of sodium in 50 ml. of dry ethanol was added with stirring 18.3 g. (0.05 mole) of diethyl carbobenzyloxyglycylaminomalonate. To the viscous yellow solution obtained after 15 minutes of stirring was added in one portion 7.1 g. of methyl iodide. The reaction mixture was then refluxed and stirred for 2 hours, another 4.0 g. of methyl iodide was added, refluxing was continued 8 more hours and then still another 3.1 g. of methyl iodide was added after which refluxing was continued for a final 5 hours. The mixture was cooled and poured into ice with stirring. The separated oil was taken up in ether, washed with water and saturated brine, and dried over anhydrous magnesium sulfate. After filtering, the solvent was evaporated, and the residual oil was dried to constant weight in a vacuum at 50°; yield 14.96 g. (79%). This was hydrogenated directly.

In extending this procedure to the preparation of higher homologs it was found that addition of the alkyl iodide (100% excess) in one portion did not affect the yield.

Method O. By a Michael Reaction of I. Preparation of Diethyl α-Cyanoethylcarbobenzyloxyglycylaminomalonate.—A freshly cut cube of sodium about one-eighth inch on a side was dissolved in 15 ml. of dry ethanol and 12.2 g. (0.0333 mole) of diethyl carbobenzyloxyglycylaminomalonate was added to give a thick slurry. To this was added with stirring 2.00 g. (0.0377 mole) of acrylonitrile dropwise over a period of ten minutes during which time some heat was evolved and the entire mass liquefied to a light yellow solution. After stirring one hour at room temperature, the solution was filtered and allowed to stand overnight at room temperature. Since the product gave no evidence of crystallizing, the solution was poured into water, the precipitated oil was taken up in ether and the ether solution was dried over anhydrous magnesium sulfate. The solvent was removed and the residual oil was dried in a vacuum at 50° to give 12.0 g. (86%) of crude product. This was hydrogenated directly.

Method P. Michael Reactions of II. Preparation of 3-Carbethoxy-3-β-cyanoethyl-2,5-piperazinedione.—A cube of sodium about one-eighth inch on a side was dissolved in 15 ml. of dry ethanol and 3.72 g. (0.02 mole) of 3-carbethoxy-2,5-piperazinedione was added. To this suspension was added 1.10 g. (0.0208 mole) of acrylonitrile dropwise with shaking. Heat was evolved and the reaction mixture set to a solid mass, which was broken up and kept at room temperature for 2 hours with occasional shaking. After removal by filtration, the colorless solid was washed three times with dry ethanol and dried to give 4.10 g. (85%) of crude product, m.p. 199–202°. Recrystallization from 125 ml. of water gave 2.90 g. of colorless plates, m.p. 207–208°. When mixed with a sample, m.p. 205–206°, of the cyano compound prepared by the reductive cyclization (method C) of diethyl β-cyanoethylcarbobenzyloxyglycylaminomalonate (method O), there was no depression of melting point.

Although substitution of ethyl acrylate for acrylonitrile in the above procedure led to the expected product in isolable form (Table IV), acrolein gave only a product in the form of a brittle, hygroscopic glass not successfully converted to a purified form.

NORTH CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF MICROBIOLOGY OF YALE UNIVERSITY]

An Ornithine–Proline Interrelation in *Escherichia coli*[1]

BY HENRY J. VOGEL

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Ornithine-C^{14}, supplied at low concentration (0.1 μg. per ml.) to growing *Escherichia coli* cells, is incorporated into the proline (and glutamic acid) as well as into the arginine of the bacterial protein. The conversion of ornithine to proline constitutes a quantitatively minor metabolic link and appears to involve glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylate as intermediates. When ornithine-C^{14} is supplied at higher concentrations, patterns of labeling result that tend to deëmphasize this ornithine-proline link.

In *E. coli*, the major biosynthetic routes leading to proline and to ornithine have been established.[2] Proline was shown to be formed from glutamate *via* glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylate[3]; and ornithine is synthesized from glutamate *via* N-acetylglutamate, N-acetylglutamic γ-semialdehyde and N^{α}-acetylornithine.[4] Studies with N-acetylglutamate-C^{14} have suggested a quantitatively minor pathway (not *via* glutamic acid) contributing to proline synthesis.[5] In the present investigation, tracer incorporation experiments with the same microbial species have demonstrated a limited conversion of *exogenous* ornithine into proline; and isotopic competition experiments have indicated that this conversion proceeds *via* glutamic γ-semialdehyde. In view of these findings, a similar conversion of *endogenous* ornithine to proline may well account for the minor pathway previously reported.

The appreciable *relative* contributions of labeled ornithine to the proline and glutamic acid of the bacterial protein that have been observed in the present incorporation experiments appear to depend on the low concentration (0.1 μg. per ml.) of the ornithine used. At higher ornithine concentrations (*e.g.*, 10 μg. per ml.) the specific activities of protein proline and glutamic acid are decreased relative to that of protein arginine. The latter amino acid is known to be derived biosynthetically from ornithine.[6] In the isotopic competition experiments of Abelson,[6] ornithine used at a still higher concentration (about 100 μg. per ml.) gave rise to almost all the protein arginine, but did not contribute detectably, within the sensitivity of the method employed,[7] to protein proline and glutamic acid.

The present incorporation experiments with uniformly C^{14}-labeled ornithine were carried out by

(1) These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Yale University.

(2) H. J. Vogel, in W. D. McElroy and B. Glass, "Amino Acid Metabolism," The Johns Hopkins Press, Baltimore, Md., 1955, p. 335.

(3) H. J. Vogel and B. D. Davis, THIS JOURNAL, **74**, 109 (1952).

(4) H. J. Vogel, *Proc. Natl. Acad. Sci.*, **39**, 578 (1953).

(5) H. J. Vogel, P. H. Abelson and E. T. Bolton, *Biochim. Biophys. Acta*, **11**, 584 (1953).

(6) P. H. Abelson, *J. Biol. Chem.*, **206**, 335 (1954).

(7) It is estimated that a contribution by exogenous ornithine to proline, amounting to 10 moles per 100 moles proline formed, could have been readily detected.