Experimental

Piperid-2,3-dione-3-phenylhydrazone.—A solution of 8.5 g. of 3-carbethoxy-2-piperidone in 100 cc. of water containing 3 g. of potassium hydroxide was kept at 30° overnight and then brought to *p*H 4–5 by the addition of dilute hydrochloric acid. It was then treated with stirring at 0° with a partially neutralized solution (150 cc.) of benzenediazonium chloride prepared from 4.85 g. of aniline and 3.75 g. of sodium nitrite, and after stirring for a few minutes the *p*H of the solution was brought to 5–6 by the addition of 40 cc. of a 45% aqueous solution of sodium acetate. A yellow turbidity formed immediately. Stirring at 5–10° (internal temperature) was continued for 5 hours, the solid filtered, washed with water and a small amount of alcohol and dried; yield 8.8 g. Recrystallization from aqueous alcohol gave pale cream-colored needles, m.p. 244–245° dec.

Anal. Calcd. for $C_{11}H_{13}ON_3$: C, 65.0; H, 6.45; N, 20.7. Found: C, 65.2; H, 6.6; N, 21.2.

3-Acetamido-2-piperidone.—1.92 g. of the above hydrazone in 30 cc. of warm glacial acetic acid was slowly added at 20–25° to a stirred suspension of 5 g. of zinc dust in 15 cc. of glacial acetic acid containing 8 cc. of acetic anhydride. Stirring at room temperature was continued for 0.5 hour, the mixture was then filtered, and the filtrate diluted with 20 cc. of water, allowed to stand for 1 hour and evaporated to dryness *in vacuo*. The residue was extracted repeatedly with ether to remove acetanilide, the residue extracted with hot chloroform, the extract taken to dryness and the residue again extracted with ether. The final residue was dissolved in hot chloroform, filtered from some insoluble material, concentrated and treated with ether and a little acetone until a cloudiness appeared. After some time the crystalline solid was filtered, washed with acetone, and dried; yield 0.55 g., m.p. 181–184°. Recrystallization from chloroform–ether gave colorless needles, m.p. 184–185° (Bergmann and Koster[3] give m.p. 187–188°).

Anal. Calcd. for $C_7H_{12}O_2N_2$: C, 53.8; H, 7.7; N, 17.9, Found: C, 53.9; H, 7.7; N, 17.4.

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A Simple Synthesis of Isotopic Citrulline and Two of Its Homologs

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A standard synthesis of citrulline from urea and copper chelated ornithine was developed by Kurtz[2] and recently was described in detail.[3] This method gives a good yield of citrulline from ornithine (approximately 60–70% of theoretical) but a low yield from urea (*ca.* 15%) because of the excess of urea required for optimal synthesis by the fusion procedure. Changing the urea to ornithine ratio to equimolar amounts gives a maximal yield of citrulline from urea of about 25%.[4] Recent interest in citrulline has been largely directed toward the reactions of its carbamyl group in arginine formation,[5] degradation by citrullinase,[6] and conversion to pyrimidines *via* ureidosuccinic acid.[7] A simple synthesis of citrulline, especially adapted to isotopic labeling of the carbamyl group, has been developed by the condensation of copper chelated ornithine with cyanate. Yields of free citrulline of 55–65% based on either ornithine or cyanate were obtained. This method would seem to offer the following advantages: (1) a high yield of carbamyl labeled citrulline from the isotopic precursor, about 2.5 times that obtained by the Kurtz procedure; (2) simplicity and rapidity, the use of a sealed tube is avoided and at 100° the reaction is complete in 20 minutes, (3) more complete conversion of copper citrulline to free citrulline.

By analogous reactions carbamyl labeled l-homocitrulline-C^{14} (ε-carbamyllysine) and 1-α-amino-γ-carbamidobutyric acid-C^{14} were synthesized and found to be inactive as substrates for citrullinase and for conversion to compound X/carbamyl phosphate. Both compounds were weak inhibitors (inhibition indices of about 10 for 50% inhibition) of the release of CO_2 from 1-citrulline-C^{14} by citrullinase, but did not inhibit compound X/carbamyl phosphate formation at this concentration. Ornithine was found to be an unusually potent inhibitor of compound X/carbamyl phosphate formation from citrulline, which will be detailed elsewhere.[7] The previously described inhibition of citrullinase by ornithine[6] was confirmed.

Experimental

Synthesis of Citrulline.—410 mg. (2.0 mmoles) of *l*(+)-ornithine dihydrochloride was chelated with cupric carbonate as described by Kurtz[8] and it reacted with 178 mg. (2.2 mmoles) of potassium cyanate at 37° in a final volume of about 3 cc. Precipitation of copper citrulline began within 2 hours. (A time study of yield based on ornithine revealed: 24 hr., 68%; 48 hr., 74.5%; 72 hr., 77%; 96 hr., 78.5%; 120 hr., 80%). Yield of crude copper citrulline was 312 mg. (75% based on ornithine). The range of yield was 72–82% on repeated tests. The copper citrulline was brought into solution with 0.8 cc. of 6 *N* HCl, diluted with 8 cc. of water and treated with H_2S for 10–15 minutes. CuS was removed by filtration, and chloride removed with a Dowex 2-acetate column (1 × 8 cm.). Free citrulline was obtained by concentration of the eluate *in vacuo* and the addition of absolute ethanol as previously described.[2,3] Yield was 210 mg. (60% based on ornithine, 80% from the copper complex); m.p. 218–219° dec. (uncor.). The range in yield of free citrulline based on ornithine was 55–65% with a range in melting points (dec.) of 214–228° for this unrecrystallized product. Mixed melting points with authentic citrulline were not depressed; $(\alpha)^{22}D$ +21° (5% solution in 1 *N* HCl), reported $[\alpha]^{25}D$ +21°.[3] Paper chromatography with phenol–water 80:20 revealed a single ninhydrin spot with an R_f 0.62.[9] By recrystallization from aqueous alcohol[3] needles were obtained with m.p. 230–232° dec., but the unrecrystallized product was adequate for biological purposes.

In an alternate method the copper ornithine–cyanate solution was heated in a boiling water-bath for 20 minutes. The yield of copper citrulline by this method had a range of 70–78% based on ornithine. The rapidity and ease of this method makes it the preferable one for non-isotopic synthesis of citrulline.

For the synthesis of citrulline-C^{14}, isotopic cyanate was formed by the fusion of urea and anhydrous potassium carbonate, as described by Williams and Ronzio.[10] 120 mg. (2.0 mmoles) of urea-C^{14} was intimately ground with 166 mg. (1.2 mmoles) of potassium carbonate and transferred to a Pyrex test-tube. Better yields were obtained by beginning the fusion at 130–140° in a metal-bath, gradually in-

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creasing the temperature to about 225° and then completing the fusion with a free flame as previously described.[10] The test-tube was cooled to room temperature, 2.25 mmoles of copper chelated *l*-ornithine in a volume of 3.5 cc. was added without attempted isolation of KCNO from the excess K_2CO_3, and the mixture kept at 37° for 5 days. Citrulline-C^{14} was isolated as described above. Yield was 179 mg. (51% based on urea) with m.p. 218–219° dec. Yields of free citrulline as high as 60% based on urea have been obtained. When this synthesis was carried out by heating at 100° for 20 minutes, the yield was usually 10–15% less than that obtained at 37°. The longer reaction period was therefore routinely used for isotopic synthesis.

Synthesis of 1-Homocitrulline·HCl (ε-Carbamyllysine).—The free form of this compound was prepared by Kurtz using the urea fusion method.[8] 365 mg. (2.0 mmoles) of *l*(+)-lysine hydrochloride was chelated with cupric carbonate as described above. Potassium cyanate (178 mg., 2.2 mmoles) was added to the solution in a final volume of 5 cc. and reacted at 37° for 4 days. The crude Cu-homocitrulline was dissolved in 2 cc. of 4 *N* HCl, diluted with 6 cc. of water and treated with H_2S for 10 minutes. The hydrochloride was crystallized from the filtrate by evaporation to dryness and the gradual addition of ethanol-ether with scratching. Yield was 330 mg. (73% based on lysine); m.p. 177–178° dec. (uncor.). Calcd. for $C_7H_{16}N_3O_3Cl$: C, 37.25; N, 18.58; Cl, 15.75. Found: C, 37.50; N, 17.87; Cl, 15.31. Paper chromatography with phenol–water (80:20) revealed a single ninhydrin spot with a R_f of 0.73. In contrast to citrulline and *l*-α-amino-γ-carbamidobutyric acid the hydrochloride of homocitrulline is easily isolated and is much more conveniently recrystallized than the fluffy, amorphous free form.

C^{14} 1-Homocitrulline was synthesized as described for C^{14} citrulline, with isolation as the hydrochloride, on a 1 mM. scale with a yield of 60% based on urea.

Synthesis of *l*-α-Amino-γ-carbamidobutyric Acid.[8]—Seven hundred and sixty-four mg. (4 mmoles) of α,γ-diaminobutyric acid dihydrochloride[11] after chelating with cupric carbonate, reacted with 356 mg. (4.4 mmoles) of potassium cyanate and was converted to the free form as described for citrulline. Yield was 245 mg. (38% based on α,γ-diaminobutyric acid); m.p. 207–208° dec. Since Kurtz reported the m.p. as 225°, an analysis was performed. Calcd. for $C_5H_{11}N_3O_3$: C, 37.30; N, 26.15. Found: C, 37.43; N, 26.18. Paper chromatography with phenol–water (80:20) revealed a single ninhydrin spot with an R_f of 0.58.

C^{14} 1-α-Amino-γ-carbamidobutyric acid was synthesized on a 1 mM. scale as described for C^{14}-citrulline with a 26% yield based on urea.

Enzyme Studies.—Isolated mitochondria from 2 rat livers were frozen in 16 cc. of distilled water and lyophilized. The dried powder (40 mg./cc.) was extracted with 0.02 molar glycylglycine at *p*H 8.0, 0°, and then centrifuged for 15 minutes at 15,000 *g*, −5°. The supernatant was used immediately for either citrullinase or compound X/carbamyl phosphate studies and contained about 1.0 to 2.0 mg. N/cc. The reactions were carried out for 20 minutes, 37°, with constant shaking in closed Warburg type flasks containing 0.6 cc. of 6 *N* KOH in the center well. One hundred μmoles of carrier $NaHCO_3$ was then added to each flask and the reaction was stopped by the addition of 0.5 cc. of 12 *N* H_2SO_4 from the side-arm. Center well bicarbonate, collected by an additional 30 min. of shaking, was precipitated as $BaCO_3$, washed repeatedly and filtered to form infinitely thick planchettes. The planchettes were counted with an end window counter having an efficiency of approximately 5%. An experimentally determined conversion factor was used to calculate citrulline degradation in μM. Details of these procedures will be published elsewhere.[7]

(a) Citrullinase.—Citrullinase activity was measured by arsenolysis of citrulline at *p*H 6.7.[6,7] Each flask contained 250 μ moles of sodium arsenate buffer, 1.0 cc. of mitochondrial extract and 5 μmoles of C^{14}-1(+)-citrulline (0.05 μc./μmole), or 5 μmoles of C^{14}-1-homocitrulline (0.2 μc./μ mole), or 5 μmole of C^{14}-1-α-amino-γ-carbamidobutyric acid (0.2 μc./μmole) in a final volume of 3.0 cc. For inhibition studies varying amounts of non-isotopic *l*(+)-ornithine, 1-homocitrulline or 1-α-amino-γ-carbamidobutyric acid were added to flasks containing 5 μmoles of isotopic citrulline.

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(b) Compound X[12] and Carbamyl Phosphate[13] Formation.—It has been demonstrated that the carbamyl group of citrulline is transferred to aspartate *via* compound X and carbamyl phosphate to form ureidosuccinate.[7] This reaction is dependent on the presence of ATP, Mg^{++} and acetylglutamate (or carbamylglutamate) and has a *p*H optimum of 8.0. In the absence of aspartate the reaction can be followed by the production of "acid labile CO_2" from citrulline at *p*H 8.0 and dependent upon the presence of acetylglutamate. Each flask contained 10 μmoles of acetylglutamate, 20 μmoles of ATP, 40 μmoles of Mg^{++}, 250 μmoles of glycylglycine buffer *p*H 8.0, 1.0 cc. of mitochondrial extract and 5 μmoles of C^{14}-1(+)-citrulline (0.1 μc./μmole), or 5 μmoles of C^{14}-1-homocitrulline (0.2 μc./μmole), or 5 μmoles of C^{14}-1-α-amino-γ-carbamidobutyric acid (0.2 μc./μmole) in a final volume of 3.0 cc. Inhibition studies were carried out as above.

Table I summarizes the enzyme studies. Homocitrulline and α-amino-γ-carbamidobutyric acid were completely inactive as substrates for these two enzymes and were weak inhibitors of citrullinase only. Ornithine caused a 50% inhibition of citrullinase in equimolar concentration, but exhibits an inhibition index of about 0.05 for compound X/carbamyl phosphate formation.

TABLE I

ENZYME DATA ON CITRULLINE AND TWO ANALOGS

		Citrullinase[b]		Compound X/carbamyl phosphate[b]	
		C.p.m.	μmole	C.p.m.	μmole
C^{14}-Homocitrulline		0	0	0	0
C^{14}ABC[a]		0	0	0	0
C^{14}-Citrulline		1320	1.37	895	0.45
+ Homocitrulline	50 μmole	790	0.82	970	.49
+ ACB[a]	50 μmole	700	.73	660	.33
+ Ornithine	5 μmole	575	.60		
	0.1 μmole			730	.37
	.2 μmole			555	.28
	.3 μmole			275	.14

[a] ABC indicates α-amino-γ-carbamidobutyric acid. [b] Results are given in counts/min. and the calculated μ mole of the C^{14} precursor this represents.

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Synthesis of Some 6-(Substituted)-Aminopurines

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Adenine and certain of its derivatives have been found to increase the number of buds from which gametophores develop in moss (*Tortella caespitosa*).[1] The structural relationship of kinetin,[2] 6-(2-furfuryl)-aminopurine (isolated from autoclaved sperm desoxyribonucleic acid as a cell division factor for tobacco callus tissue) to these compounds which increase budding in moss, led to the preparation and testing of kinetin and a group of its analogs in this Laboratory.

All of the analogs were synthesized by the general procedure of Elion, Burgi and Hitchings[3] whereby 6-methylmercaptopurine is condensed with the appropriate amine at elevated temperatures, as indicated in Table I.

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