until the crystals dissolved (10 minutes). After an additional 5 minutes the solution was acidified with dilute hydrochloric acid and extracted with ether. The combined ether layers were washed with saturated sodium bicarbonate solution and dried over anhydrous magnesium sulfate. The residue obtained on evaporation of the solvent at reduced pressure was chromatographed on 4 g. of Florex. The fraction (0.034 g.) eluted with benzene was crystallized from ether to give 0.022 g. of colorless platelets, m.p. 132–133°. Repeated recrystallization from ether and from petroleum ether (65–68°) did not alter the m.p.

Anal. Calcd. for $C_{20}H_{26}O_3$: C, 76.40; H, 8.34. Found: C, 76.3; H, 8.27.

Enol Acetate of Estrone Acetate.—A solution of 1.00 g. of estrone, m.p. 253–257°, and 0.5 g. of *p*-toluenesulfonic acid in 50 ml. of isopropenyl acetate was distilled slowly (high reflux ratio) through a 5-in. Vigreux column for 17 hr. The crude product, isolated as described above for the preparation of II, was adsorbed on a column of 30 g. of Florex. The early fraction (0.99 g.) eluted with 10% benzene in petroleum ether (65–68°) gave, on crystallization from methanol, 0.87 g. of colorless elongated flat prisms, m.p. 149–151°. Further recrystallization did not raise this m.p.

Anal. Calcd. for $C_{22}H_{26}O_4$: C, 74.55; H, 7.39. Found: C, 74.3; H, 7.52.

After the completion of this preparation, Leeds, Fukushima and Gallagher[14] published the preparation of this substance, m.p. 149–150°, by an improved procedure.

16-Bromoestrone Acetate.—A solution of 0.045 g. of the aforementioned enol diacetate, m.p. 149–151°, in 1 ml. of carbon tetrachloride was treated at 0° with 0.0184 g. of bromine in 1.2 ml. of carbon tetrachloride. Within 3 minutes the solution was colorless, and the solvent was evaporated at reduced pressure. Trituration of the residue with ether gave 0.038 g. of crystals, m.p. 163–168°. Repeated recrystallization from methanol gave colorless rods, m.p. 168–170°.

Anal. Calcd. for $C_{20}H_{23}O_3Br$: C, 61.38; H, 5.92. Found: C, 61.0; H, 5.87.

MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, THE UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

A New Synthesis of DL-Lysine-1-C^{14}

BY MORTON ROTHSTEIN[1]

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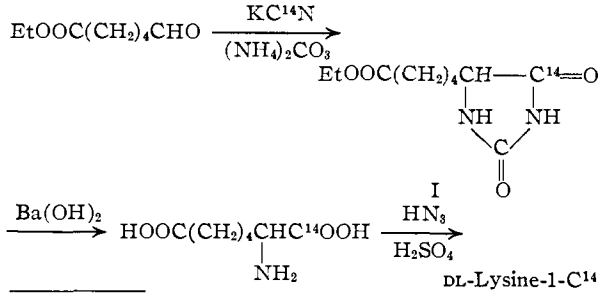
DL-Lysine-1-C^{14} has been synthesized in an over-all yield of 66.5% based on KC^{14}N. An 86% yield of the intermediate DL-α-aminopimelic acid-1-C^{14} was obtained.

DL-Lysine-1-C^{14} has previously been synthesized in two ways. Borsook, *et al.*,[2] using the method of Gaudry,[3] prepared 5-δ-bromobutylhydantoin-4-C^{14} from KC^{14}N. Treatment with ammonia followed by hydrolysis of the resulting amino hydantoin yielded lysine-1-C^{14} in 10–15% yield. Barry[4] reports a simplified procedure with an increased yield. Arnstein, *et al.*,[5] utilized the carboxylation of cyclohexanone with $C^{14}O_2$ in liquid ammonia to form 2-oxocyclohexanecarboxylic acid. Esterification of the product, followed by treatment with hydrazoic acid, led to lysine-1-C^{14} in an over-all yield of 12%. Both methods suffer from low over-all yields and rather long procedures. For this reason, an entirely new synthesis was undertaken which would provide a simple method for the preparation of DL-lysine-1-C^{14} in good yield. The following equations show the synthetic route selected



(1) This work was supported by Research Grant C-2327 of the U. S. Public Health Service and the Hobson Fund of the Cancer Research Institute, University of California School of Medicine.

(2) H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley and P. H. Lowy, *J. Biol. Chem.*, **184**, 529 (1950).

(3) R. Gaudry, *Can. J. Res.*, **26B**, 387 (1948).

(4) J. M. Barry, *J. Biol. Chem.*, **195**, 795 (1952).

(5) H. R. V. Arnstein, G. D. Hunter, H. M. Muir and A. Neuberger, *J. Chem. Soc.*, 1329 (1952).

In two cold runs, using equimolar amounts of aldehyde and potassium cyanide, the yields of the hydantoin I were 51 and 53%, respectively. A large excess of aldehyde does not improve the yield based on cyanide but complicates the isolation of pure hydantoin. Freshly prepared aldehyde should be used, since use of material several days old reduced the yield of I to 39%. Matched runs using sodium cyanide and potassium cyanide gave only 46% of I in the former case, compared to 53% in the latter. Preparation and isolation of the cyanohydrin of ethyl 5-formylvalerate, followed by treatment of the product with ammonium carbonate, gave the same yield of I as the one-step procedure.

In the "hot run," only 18.6% of I could be isolated from the reaction mixture. However, hydrolysis of the mother liquors with barium hydroxide yielded an additional 67.5% of DL-α-aminopimelic acid-1-C^{14}, making the over-all yield of this compound 86%, based on KC^{14}N. Apparently, an α-aminopimelic acid precursor existed in the mother liquors, possibly the hydantoin amide or potassium salt derived from I. The only apparent difference between the radioactive run and the cold runs was the presence of an excess of potassium hydroxide in the KC^{14}N solution. The excess alkali was partly neutralized with hydrochloric acid before proceeding with the reaction. Thus, it would seem likely that even in the "cold runs," where a 53% yield of hydantoin was obtained, a considerable amount of this α-aminopimelic acid precursor was present.

The isolation of α-aminopimelic acid-1-C^{14} after hydrolysis of the mother liquors of the Bucherer reaction was complicated by the presence of inorganic salts from the original reactants. The acid

was therefore isolated by passage of the hydrolysate through a strong base anion exchange resin (Dowex-2). Elution with 6 *N* acetic acid, followed by evaporation of the eluates, yielded a salt-free product.

Conversion of α-aminopimelic acid to lysine was carried out with hydrazoic acid by the procedure of Adamson.[6] However, small changes in the procedure increased the yield from a reported 66 to 77.5%. The increase may be due in part to the simplified procedure for the isolation of lysine which was devised, making use of the ion exchange resin Dowex-50, and thus eliminating the need for intermediate precipitations (phosphotungstate) or derivatives (picrate).

The chemical yield of lysine-1-C^{14} from $KC^{14}N$ was 66.5%. The specific activity of the product was nearly 20% lower than calculated, but this was probably due to losses of activity during the concentration of the $KC^{14}N$ solutions or other errors in assaying the $KC^{14}N$. The cyanide used in this synthesis was a combination of preparations from several sources.

Experimental

5-(4-Carbethoxybutyl)-hydantoin: A (From the Aldehyde).—To 2.4 g. (15 mmoles) of freshly prepared ethyl 5-formylvalerate[7] in a solution of 20 ml. of 95% ethanol and 20 ml. of water, was added 1.02 g. of potassium cyanide (95% min. purity, 15 mmoles) and 5.8 g. of powdered ammonium carbonate. The mixture was stirred overnight at 50–60° and then boiled down to a volume of approximately 20 ml. The solution was cooled overnight in the refrigerator and the resulting white solid was filtered, washed with a little cold water and finally with a little cold ether. The yield was 1.840 g. or 53.5% of white solid, m.p. 125–126°. The mother liquors yielded a light yellow oil after concentration. An analytical sample was prepared by recrystallization from water.

Anal. Calcd. for $C_{10}H_{16}O_4N_2$: C, 52.63; H, 7.07. Found: C, 52.72; H, 7.00.

5-(4-Carbethoxybutyl)-hydantoin: B (From the Cyanohydrin).—A solution of 2.05 g. of potassium cyanide in 10 ml. of water was added to a clear solution of 4.74 g. of aldehyde and 3.12 g. of sodium bisulfite in 40 ml. of water. During stirring for 45 min., a colorless oil formed on the surface. The mixture was extracted three times with ether and the extracts were dried over sodium sulfate, filtered and evaporated. The residual oil was poured into a solution of 9 g. of ammonium carbonate in 80 ml. of ethanol–water (1:1).

The product was isolated as above, yielding 2.67 g. (39%) of the hydantoin. A preparation run concomitantly with the same batch of aldehyde (several days old) but using procedure A also gave a 39% yield of product.

5-(4-Carbethoxybutyl)-hydantoin-4-C^{14}.—A solution calculated to contain 12.2 mM. of $KC^{14}N$ (21 mc.) and an estimated 12 mmoles of potassium hydroxide was transferred to a 200-ml. 3-necked flask and made up to 30 ml. with water; 30 ml. of ethanol was added and the solution was cooled in an ice-bath. The solution was stirred, and 573 mg. (8.8 mmoles) of inert potassium cyanide was added, followed by 1.5 ml. of cold 6 *N* hydrochloric acid. Then 8 g. of ammonium carbonate and 3.5 g. of freshly prepared ethyl 5-formylvalerate were added with stirring. The mixture was stirred for 10 hr. at 50–60°. A sodium hydroxide trap to collect vapors from the reaction mixture showed negligible radioactivity after 4 hr. and was discontinued. The reaction mixture was treated as in A above, with due care. This led to three crops of 5-(4-carbethoxybutyl)-hydantoin-4-C^{14}, totaling 0.891 g. (18.6%). The ether and water washes were combined with the mother liquors.

(6) D. W. Adamson, *J. Chem. Soc.*, 1564 (1939).

(7) G. B. Brown, M. D. Armstrong, A. W. Moyer and W. P. Anslow, Jr.; B. R. Baker, M. V. Querry, S. Bernstein and S. R. Safir, *J. Org. Chem.*, **12**, 160 (1947).

DL-α-Aminopimelic Acid-1-C^{14}.—The hydantoin was transferred to a heavy-walled glass tube with the aid of a little methanol, and the methanol was evaporated with a stream of air. The residue was dissolved in 20 ml. of hot water, 3.0 g. of anhydrous barium hydroxide was added and the tube sealed off. After heating for 24 hr. at 130°, the tube was cooled, opened and the contents washed into a 500-ml. erlenmeyer flask. The mixture was adjusted to approximate neutrality with sulfuric acid, and then just enough extra acid was added to bring the *p*H to 2. Celite "Filter Aid" was added, the mixture was swirled vigorously and then allowed to stand for several hours. After filtration, the residue was washed thoroughly with hot water, and the washes were added to the filtrate.

The filtrate was evaporated to about 20 ml. and 40 ml. of ethanol was added. Then a slight excess of pyridine was added, followed by 15 ml. of ether. After standing overnight in the cold, the crude α-aminopimelic acid-1-C^{14} was filtered, washed with ethanol and finally with ether. The mother liquors were evaporated to dryness and treated with 10–15 ml. of ethanol. The insoluble material was filtered and added to the main crop of product.

Dissolution in water, filtration and precipitation with ethanol yielded 687 mg. (100%) of α-aminopimelic acid-1-C^{14}. The material gave only one ninhydrin positive spot on paper chromatograms when co-chromatogrammed with authentic α-aminopimelic acid. The spot corresponded to the only radioactive area.

α-Aminopimelic Acid from the Mother Liquors of 5-(4-Carbethoxybutyl)-hydantoin-4-C^{14}.—The mother liquors were evaporated to a thick oil and hydrolyzed as above in two portions, and then the portions were combined.

After removal of barium sulfate as above, the filtrate was concentrated and split into four portions. Each was passed through a 4" × 7/8" column of Dowex-2 (OH^-) anion exchange resin and washed with water until the eluate was no longer basic. The α-aminopimelic acid-1-C^{14} was then eluted with 6 *N* acetic acid. The combined acetic acid eluates were evaporated to dryness, and the residue was dissolved in a minimum of warm water, filtered and treated at 70° with twice the volume of ethanol. The white crystalline product (2 crops) weighed 2.48 g. The total yield of α-aminopimelic acid-1-C^{14}, based on cyanide, was 86%, including the acid obtained from hydrolysis of the hydantoin.

DL-Lysine-1-C^{14}.—The α-aminopimelic acid-1-C^{14} was converted to lysine-1-C^{14} in portions of 0.687 and 2.51 g., respectively. From the smaller run, 0.03 g. of unreacted α-aminopimelic acid-1-C^{14} was recovered and combined with the second run. The procedure of Adamson[6] was followed, except that concentrated sulfuric acid was replaced by 100% sulfuric acid, and an additional excess of hydrazoic acid was used (1.5:1 molar ratio instead of 1.2:1). After dilution of the sulfuric acid and removal of the chloroform, the solution was neutralized with barium hydroxide and then made just acid with dilute sulfuric acid. Celite Filter-aid was added and the mixture allowed to stand for several hours. After filtration, the combined filtrate and washes of the residue were evaporated to a small volume and run slowly through a 6" × 7/8" column of Dowex-50 (H^+) sulfonic acid ion exchange resin. The column was washed with water until the eluate was neutral and gave no test for $SO_4^=$ and then treated with 1 *N* hydrochloric acid. From the small run, 30 mg. of unreacted α-aminopimelic acid-1-C^{14} first passed out of the column. When the eluate was ninhydrin negative and non-radioactive, elution was continued with 2 *N* hydrochloric acid and lysine-1-C^{14} was collected in the eluate. The last of the lysine was removed from the resin with 6 *N* acid. No α-aminopimelic acid was obtained from the larger run.

The lysine solutions were evaporated to dryness and the resulting dihydrochlorides converted to monohydrochlorides with pyridine in the usual manner.[8] The yields of once recrystallized DL-lysine-1-C^{14} were 66 and 70% (0.383 and 1.76 g.), respectively, taking into account the recovered α-aminopimelic acid from the smaller run. The mother liquors from the pyridine precipitation and lysine recrystallizations were combined and passed through the column of Dowex-50 once again. After treatment as above, an

(8) "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1941, p. 375.

additional 483 mg. of DL-lysine-1-C^{14} was obtained, making a total average yield of 77.5% for the two Schmidt reactions. The over-all yield of pure DL-lysine-1-C^{14} from $KC^{14}N$ was 66.5%.

All of the portions of DL-lysine-1-C^{14} monohydrochloride were chemically and radioactively pure as shown by paper chromatography and radioautography; m.p. 263–264° dec.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

Formation of Schiff Bases of Pyridoxal Phosphate. Reaction with Metal Ions[1]

BY YOSHIHIKO MATSUO

RECEIVED OCTOBER 10, 1956

From a spectrophotometric study of the reactions of pyridoxal phosphate (PLP) with amino acids and amines evidence was secured for the formation in neutral aqueous solution of Schiff bases between PLP and various amino compounds. The stability constants of the Schiff bases were evaluated by an optical method. Spectrophotometric evidence was obtained of the chelation of the PLP Schiff bases with certain metal ions. It was found that chelating metal ions can be removed from some metal–PLP–amino acid complexes by suitable concentrations of ethylenediaminetetraacetic acid or 2,3-dimercaptopropanol. The significance of the findings is discussed in connection with the mechanism of reactions of vitamin B_6 enzymes.

During investigation of homoserine deaminase of rat liver, the activity of which is significantly increased by the addition of pyridoxal phosphate (PLP), it was noted that addition of homoserine to a dilute neutral solution of PLP (10^{-5} M) caused a rapid intensification of the yellow color of the mixture. This was observed with many other amino acids and also with several primary amines. However, N-substituted amino acids, such as sarcosine, did not cause the color change. From this it was inferred that the intensification of the yellow color of PLP on addition of amino compounds is due to the formation of Schiff base. It is known that Schiff base is formed readily between pyridoxal and amino acids,[2,3] or pyridoxal and amines,[4,5] both in an alcoholic medium[2-4] and in aqueous solution.[5] Roberts[6] has observed a change in the absorption spectrum of PLP in the presence of glutamic acid or alanine, and suggested that this shift in the spectrum might be due to the formation of Schiff base. More recently, while the present work was in progress, Blakley[7] reported the ultraviolet spectra of such bases formed between PLP, and glycine and serine, respectively. He also showed that the presence in the reaction mixture of bisulfite, which blocks the formyl group of PLP, prevents such change in spectrum on addition of the amino acids. Since the formation of Schiff base is generally believed to be involved in the mechanism of reaction catalyzed by vitamin B_6 enzymes, a more detailed study was made on the chemistry of this reaction between PLP and amino acids and the results are reported in this paper. Interaction of metal ions and the Schiff base was also studied to a limited extent.

(1) Aided by research grants from the National Cancer Institute (No. 327 and 2327), the American Cancer Society (Met-45A), and the Cancer Research Funds of the University of California.

(2) D. Heyl, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3429 (1948).

(3) H. Brandenberger and P. P. Cohen, *Helv. Chim. Acta*, **36**, 549 (1953).

(4) D. Heyl, E. Luz, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3669 (1948).

(5) V. R. Williams and J. B. Neilands, *Arch. Biochem. Biophys.*, **53**, 56 (1954).

(6) E. Roberts, *J. Biol. Chem.*, **198**, 495 (1952).

(7) R. L. Blakley, *Biochem. J.*, **61**, 315 (1955).

(1) Reaction of Pyridoxal Phosphate with Amino Acids and Amines.—Addition of amino acids or primary amines to a solution of PLP prepared in a potassium phosphate buffer, *p*H 7.5, caused a rapid and marked intensification of the yellow color of the solution. Upon measurement of the ultraviolet absorption spectra of PLP in the presence and absence of amino acids or amines it was observed, as previously reported by other workers,[6,7] that the spectrum is significantly altered by the addition of amino acids or amines. This is illustrated in Fig. 1. PLP has two absorption maxima; one at 330 mμ and the other at 388 mμ. In the presence of various amino compounds, a new maximum appeared at 278 mμ, and the 388 mμ peak moved to a higher wave length (400 to 415 mμ), while the maximum at 330 mμ decreased in intensity and was reduced to an inflection point. Spectral changes of this type were observed on addition of the following compounds to the solution of

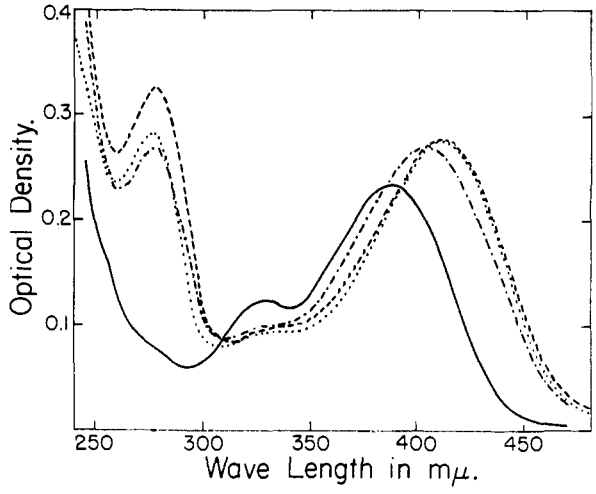

Fig. 1.—Spectrum of pyridoxal phosphate (4.30×10^{-5} M) in 0.17 M potassium phosphate buffer, *p*H 7.5: —, alone; - - - -, plus 8×10^{-2} M α-amino-*n*-butyric acid; . . . ., plus 8×10^{-2} M ethanolamine; -·-·-, plus 8×10^{-2} M α-methyl-α-amino-*n*-butyric acid. All the amino compounds neutralized to *p*H 7.5 before addition.