identical with material obtained by similar reduction of *dl*-18-nor-D-homoepiandrosterone[1c] and is also readily prepared by direct reduction of the 13,14-dehydro precursors. *dl*-IX is devoid of significant androgenic activity, but exhibits about one-fifteenth of the myotrophic activity of *d*-testosterone.

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CARBAMYL PHOSPHATE, THE CARBAMYL DONOR IN ENZYMATIC CITRULLINE SYNTHESIS[1]

Sir:

The recent work on a phosphorolysis of citrulline in microbial extracts by Knivett,[2] by Slade[3,4] and by Korzenovsky and Werkman[5,6] and more recently by Stulberg and Boyer[7] has greatly advanced the understanding of the mechanism of this reaction. It seemed of considerable promise now to attempt the identification of the probable phosphorylated intermediary in this system which appeared to have a certain similarity to the so-called phosphoroclastic reaction of pyruvate in microbial extracts. In attempts to identify a phosphorylated intermediary, using extracts of *Streptococcus fecaelis* R, no reaction between ATP[8] and ornithine could be observed. As shown in Table I, however, on incubation of an equilibrium mixture of ammonium carbonate-carbamate with ATP, or better, phosphopyruvate + ADP, a relatively stable phosphorylated compound was formed. The compound decomposes only slowly in the Fiske and SubbaRow phosphate reagent, but hydrolyzed completely on one minute heating with 0.01 normal hydrochloric acid to 100° and is determined in this manner.

This precursor of the carbamyl group in citrulline has been identified by synthesis as carbamyl phosphate. Carbamyl phosphate is surprisingly easily prepared by mixing dihydrogen phosphate with cyanate in the following manner: 0.1 mole of potassium dihydrogen phosphate and 0.1 mole of potassium cyanate were dissolved in 100 milliliters of water, the solution warmed to 30° for 30 minutes, and then cooled in ice. To the cool solution, an ice-cold solution of 0.3 mole of lithium hydroxide and 0.2 mole of perchloric acid in 83 milliliters of water were added slowly, final *p*H 8.3. A precipitate forms which consists of potassium perchlorate and lithium phosphate. This is removed by filtration. The supernate contains the lithium carbamyl phosphate. This is precipitated by slow addition of an approximately equal volume of ethanol. On reprecipitation with ethanol, dilithium carbamyl phosphate of a purity of 90 to 95 per cent. was obtained which was used for enzymatic tests.

TABLE I

FORMATION OF CARBAMYL PHOSPHATE FROM ATP

The complete incubation mixture for the formation of carbamyl phosphate consisted of: 200 μM. tris-(hydroxymethyl)-aminomethane buffer, *p*H 8.5; 5 μM. $MgCl_2$; 25 μM. KF; 0.6 μM. of ADP, *p*H 7.0; 100 μM. ammonium carbonate; 5.1 μM. phosphoenol pyruvate; 10 μM. L-ornithine; 0.01 mg. crystalline pyruvate kinase; and 0.5 mg./ml. of *Streptococcus fecaelis* extract in 1 ml. final volume. Vessels were incubated at 30° for 30 minutes. Carbamyl phosphate is that phosphorus which is hydrolyzed by 0.01 *N* HCl in 1 minute at 100°. Citrulline was determined according to Archibald.[9]

		P_i, μM./ml.	P_u, μM./ml.	Citrulline, μM./ml.
1	No enzyme	0.20	0.41[a]	0
2	No phosphoenol pyruvate	0.15	0.05	0
3	No Mg or ornithine	0.32	0.46[a]	0
4	No ornithine	0.45	1.30	0
5	Complete	5.50	0.10	5.1

[a] This blank shows that our hydrolysis procedure decomposes a small fraction of the phosphoenol pyruvate.

The synthetic compound behaved analogously to the enzymatically formed compound with regard to acid hydrolysis and relative stability in the Fiske–SubbaRow molybdate mixture. Citrulline formation from synthetic carbamyl phosphate and ornithine with the microbial enzyme are shown in Table II. It may be noted that a small part of the compound decomposed spontaneously in the absence of enzyme or in its presence if ornithine is omitted. From observations of the previous workers on the phosphorolytic split of citrulline in the presence of ADP with the formation of ATP, the intermediary was expected to react easily with ADP. This is confirmed in the experiment shown in Table III, which shows a rapid reaction. We therefore formulate citrulline synthesis as

$$H_2N\cdot\overset{O}{\overset{\|}{C}}\cdot OH + ADP\cdot O\sim PO_3^- \longleftrightarrow H_2N\cdot\overset{O}{\overset{\|}{C}}\cdot O\sim PO_3^- + ADP \quad (1)$$

$$H_2N\cdot\overset{O}{\overset{\|}{C}}\sim O\cdot PO_3^- + NH_2\cdot(CH_2)_3\cdot CHNH_2\cdot COOH \longleftrightarrow H_2N\cdot\overset{O}{\overset{\|}{C}}\sim NH\cdot(CH_2)_3\cdot CHNH_2\cdot COOH + HO\cdot PO_3^- \quad (2)$$

Magnesium ion is required in reaction (1) but not in (2) (*cf.* Tables III and II).

Experiments with mitochondria have shown that CAP in the animal system likewise donates carbamyl to ornithine. The carbamyl-ornithine kinase appears more stable and far more active than the over-all reaction starting with ATP. In the

(1) This investigation was supported by research grants from the Cancer Institute of the National Institutes of Health, Public Health Service and the Life Insurance Medical Research Fund.

(2) V. A. Knivett, *Biochem. J.*, **50**, XXX (1952); **58**, 480 (1954).

(3) H. D. Slade and W. C. Slamp, *J. Bact.*, **64**, 455 (1952).

(4) H. D. Slade, *Arch. Biochem. Biophys.*, **42**, 204 (1953).

(5) M. Korzenovsky and C. H. Werkman, *ibid.*, **41**, 233 (1952).

(6) M. Korzenovsky and C. H. Werkman, *Biochem. J.*, **57**, 343 (1954).

(7) M. P. Stulberg and P. D. Boyer, THIS JOURNAL, **76**, 5569 (1954).

(8) The following abbreviations are used: ATP for adenosine triphosphate; ADP, adenosine diphosphate; CAP, carbamyl phosphate; P_i, orthophosphate; P_u, unstable phosphate; and P_{10}, phosphate hydrolyzed in 10 minutes with *N* HCl at 100°.

(9) R. M. Archibald, *J. Biol. Chem.*, **156**, 121 (1954).

TABLE II

SYNTHESIS OF CITRULLINE FROM CARBAMYL PHOSPHATE

The complete incubation mixture for the carbamyl phosphate system contained: 200 μM. tris-(hydroxymethyl)-aminomethane buffer, *p*H 8.5; 10 μM. ornithine, *p*H 8.0; 5 μM. $MgCl_2$; 6.2 μM. CAP (containing 0.8 μM. orthophosphate); and 0.1 mg. protein as the *Streptococcus faecalis* extract in 1 ml. final volume. The vessels were incubated at 30° for 30 minutes.

	P_i, μM./ml.	CAP, μM./ml.	Citrulline, μM./ml.
Complete, zero time	0.8	6.20	0
Complete, incubated	6.9	0	6.3
No Mg	6.8	0	6.3
No ornithine	2.4	4.7	0
No enzyme	2.3	4.5	0

TABLE III

FORMATION OF ATP FROM CARBAMYL PHOSPHATE

The complete incubation mixture contained: 200 μM. tris-(hydroxymethyl)-aminomethane buffer, *p*H 8.5; 10 μM. $MgCl_2$; 7.20 μM. of ADP, *p*H 7.0; 8.58 μM. of CAP (containing 1.3 μM. inorganic phosphate and 1.5 μM. ammonia[a]); 0.5 mg. protein as the *Streptococcus faecalis* extract in 1 ml. final volume. Vessels were incubated at 30° for 20 minutes. The difference figures represent the difference between the complete system deproteinized at zero time and the reaction vessels.

	P_i, μM./ml.	ATP, P_{10}, μM./ml.	CAP P_i, μM./ml.	CAP NH_3,[a] μM./ml.
Complete	+0.30	+7.4	−7.35	+7.5
No enzyme	+1.20	0	−1.64	0
No ADP	+1.30	0	−1.32	...
No Mg	+0.86	+3.4	−4.54	+3.2

[a] Ammonia was determined by a Conway distillation procedure[10] using saturated K_2CO_3 to liberate the ammonia from solution. The data reflect the stability of the fixed carbamate to alkali (*cf.* ref. 11).

experiment of Table IV, a rapid transfer from CAP is shown to occur while in the same extract, not shown in the table, ATP was inactive. With intact mitochondria, ATP showed in 60 minutes only about one-fifth of the activity shown by CAP in 10 minutes. With similar preparations, Grisiolia and Cohen[12] have previously reported on an unstable precursor of the carbamyl group in citrulline.

TABLE IV

Sonorated extract of rat liver mitochondria, 0.1 ml. (0.9 mg. protein), in 1 ml. total volume, *p*H 7.5, incubated 30 minutes at 37°. Otherwise conditions were similar to those in Table II.

	P_i, μM./ml.	CAP, μM./ml.	Citrulline, μM./ml.
Complete, 0 minutes	1.0	4.2	0
Complete, incubated	5.2	0	4.1
No CAP, incubated	0.1	0	0

If aspartic acid is substituted for ornithine in the microbial extracts as carbamyl acceptor, a somewhat slower reaction with carbamyl phosphate is observed, indicating an analogous mechanism for the synthesis of carbamyl aspartate. Formation of this compound from aspartate with ATP and ammonium carbonate or an unstable carbamyl precursor has recently been described in mammalian liver extracts by Lowenstein and Cohen[13] and by P. Reichard.[14]

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(14) P. Reichard, *Acta Chem. Scand.*, **8**, 795 (1954).

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CANESCINE AND PSEUDOYOHIMBINE FROM THE ROOTS OF *RAUWOLFIA CANESCENS* L.[1]

Sir:

Four alkaloids, α-yohimbine (rauwolscine),[2] yohimbine,[3] serpentine[3] and reserpine,[4] have so far been isolated from the roots of *Rauwolfia canescens* L. Reserpine was first discovered in *Rauwolfia serpentina*, and the hypotensive and sedative effects of rauwolfia have been mainly attributed to it.

We have now isolated two further alkaloids in pure form from the methanol mother liquor of reserpine, obtained from roots of *Rauwolfia canescens*,[5] by means of chromatography with aluminum oxide and fractional crystallization.

One of the two alkaloids, which crystallizes from methanol in hexagonal plates free from solvent, has been found to be identical with pseudoyohimbine which was originally found in yohimbé bark,[6] m.p. 265–278° (cor., in vacuum tube) with decomposition,[7] $[\alpha]^{20}D$ +27 ± 2° (*c* 0.3 in pyridine); $C_{21}H_{26}O_3N_2$ (354.4) (calcd.: C, 71.16; H, 7.39; N, 7.90. Found: C, 71.08; H, 7.56; N, 8.21). Molecular weight determined by potentiometric titration with 0.1 *N* HCl was 358. The hydrochloride crystallizes from alcohol in needles containing solvent, m.p. 250–260 (cor.) with decomposition, $C_{21}H_{26}O_3N_2 \cdot HCl$ (calcd.: C, 64.52; H, 6.96; Cl, 9.07. Found: C, 64.52; H, 7.02; Cl, 9.66). In Keller's color reaction with glacial acetic acid containing ferric chloride, and concentrated H_2SO_4, the alkaloid yields a brownish-violet stain like that of yohimbine. The ultraviolet spectrum in ethanol reveals maxima at 226 mμ and 280 mμ and a small peak at 291 mμ.

For the second alkaloid, which is not identical with any known compound, we propose the name *canescine*. It crystallizes from 15 parts of methanol

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(7) Our thanks are due to Prof. M. M. Janot and Dr. R. Goutarel, of Paris, for making available an authentic sample of pseudoyohimbine and for verifying the identity of our sample of pseudoyohimbine.