acid" was not inhibited by versene or by aerobic conditions and did not require adenosine triphosphate. Formic acid, formyl-*iso*glutamine[1], -glutamine[1] and -glutamic acid[1] were inactive, while urocanic acid was as active as FAG only at high concentrations (5 μM) and histidine was still less active*. The superiority of FAG to urocanic acid or histidine as formyl donor was clearly demonstrated when formic acid-^{14}C (25 μM) was incubated with pigeon liver extracts under the conditions used by GOLDTHWAIT *et al.*[8] for the enzymic synthesis of formylglycinamide ribotide. After rigorous removal of all volatile radioactive material 148 counts/min, corresponding to a utilization of 0.5 μM of formate, were found in the nonvolatile fraction in the presence of formic acid alone. Upon addition of 10 μM each of nonlabeled histidine, urocanic acid, FAG, formyl*iso*glutamine, formylglutamine, formylglutamic acid, formylglycine, formamidinoacetic acid[3], and γ-benzyl-FAG[3], 120, 80, 48, 135, 126, 137, 155 and 125 counts/min, respectively, were found in the nonvolatile fraction. The increasing inhibition of incorporation of formic acid into the nonvolatile fraction as one proceeds from histidine to FAG as well as the lack of inhibition by other formyl derivatives gives strong support to the role of FAG as source of the formyl group. The extent of inhibition shows that FAG does not act as a formylating agent through the production of formate. The same conclusion was reached when labeled histidine-2-^{14}C** (2.5 μM) was incubated under the above conditions in the absence or presence of a 4-fold excess of nonlabeled formate. After deproteinization, histidine was removed on a Dowex-2-formate column and the eluate brought to dryness and counted. In the presence of formate an inhibition of incorporation of ^{14}C of only 40% was observed in the nonvolatile, HCl labile[8], fraction.

The mechanism of the metabolic utilization of FAG as formyl donor as well as the detailed requirements of the soluble enzyme system are under study.

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* While this work was in progress K. SLAVIK AND V. MATOULKOVA (*Collection Czechoslov. Chem. Commun.*, 19 (1954) 1032) reported the formation of formylfolic acid, identified by paper chromatography, upon incubation for 22 hours of concentrated liver homogenates with folic acid and histidine or urocanic acid or an ionophoretically separated enzymic degradation product of histidine assumed to be FAG. In stimulating formylfolic acid formation the FAG preparation proved to be superior to histidine or urocanic acid and other formyl compounds were found to be inactive.

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On the nature of the carbamyl group donor in citrulline biosynthesis*

Optimum conditions for the biosynthesis of citrulline from ornithine, ammonia, and carbon dioxide by soluble mammalian liver enzymes require ATP**, magnesium ions and catalytic amounts of certain derivatives of glutamic acid[1]. The catalytic properties of the glutamate derivatives have been ascribed to the formation of an unstable carbamyl intermediate (Compound X) containing the glutamate derivative, ammonia, carbon dioxide and phosphate in a mole-to-mole ratio[1,2,3]. Transfer of the carbamyl group from Compound X to ornithine to form citrulline was considered to result in the regeneration of the glutamate derivative and the release of inorganic phosphate.

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** The following abbreviations are used: ATP, adenosine triphosphate; AG, acetyl glutamate; CG, carbamyl glutamate; AGI and CGI, intermediates formed in the presence of AG and CG respectively.

Following the demonstration of citrulline synthesis in bacterial extracts[4,5] one of us[6] in collaboration with Dr. V. A. KNIVETT obtained evidence for the formation of a phosphorylated intermediate in citrulline synthesis by extracts of *Streptococcus faecalis* (ST), National Collection of Type Cultures No. 6782. Since no glutamate derivative was required for the formation of this intermediate from ammonia, carbon dioxide and ATP by the bacterial system it was believed to be carbamyl phosphate. During the course of studies bearing on the nature of this intermediate JONES, SPECTOR AND LIPMANN[7] reported the formation of a phosphorylated carbamyl group precursor by extracts of *Streptococcus faecalis* R and identified it by synthesis as carbamyl phosphate. These discoveries and the availability of a ready synthesis of carbamyl phosphate led us to re-examine the nature of Compound X. One of us[3,6] had isolated and purified cyclohexylammonium salts of Compound X from incubation mixtures containing either CG or AG. Employing methods which were quantitative for AG we were unable to detect any AG in a purified sample of Compound X which had been formed in the presence of AG. Further, if the isolated intermediates formed in the presence of AG, or CG, were added to a liver enzyme system capable of synthesizing citrulline from ornithine in the presence of catalytic amounts of AG, or CG, only a stoichiometric conversion of the added intermediate to citrulline occurred (Table I). Had a glutamate derivative been a part of the Compound X added it should have been regenerated when Compound X was converted to citrulline. The regenerated glutamate derivative should then have reacted catalytically to form more than a stoichiometric amount of citrulline.

TABLE I

THE EFFECT OF GLUTAMATE DERIVATIVES AND COMPOUND X ON CITRULLINE SYNTHESIS

Final concentrations of substrates were as follows: ATP, $5\cdot10^{-3}\,M$; NH_4Cl, $2\cdot10^{-2}\,M$; $NaHCO_3$, $2\cdot10^{-2}\,M$; $MgCl_2$, $1\cdot10^{-2}\,M$; L-ornithine, $1\cdot10^{-2}\,M$, phosphate buffer, pH 7.4, $1\cdot10^{-2}\,M$. 2.08 mg of protein were added as an aqueous extract of acetone dried washed rat liver residue. Incubation at 38° for 45 minutes. Final volume = 2.0 ml. The quantity of intermediate added was estimated enzymically[2] on a separate aliquot.

Exp.	*Additions* *μM*	*Citrulline formed* *μM*
1	0.36 AGI*	0.37
2	0.36 AGI + 0.50 *μM* AG	3.80
3	1.76 AGI	1.84
4	1.76 AGI + 1.00 *μM* AG	5.38
5	0.50 AG	3.75
6	1.00 AG	3.91
7	1.00 CGI**	0.94
8	1.00 CG	1.98

* Crystalline cyclohexylammonium salt: purity[6] = 1.90 *μM*/mg.

** Cyclohexylammonium salt, purity[3] = 1.64 *μM*/mg.

TABLE II

CATALYTIC ROLE OF GLUTAMATE DERIVATIVES IN THE FORMATION OF CARBAMYL PHOSPHATE

Final concentrations of substrates were as follows: ATP, $4\cdot10^{-3}\,M$; NH_4HCO_3, $5\cdot10^{-2}\,M$; $MgCl_2$, $1\cdot10^{-2}\,M$; D-3-phosphoglyceric acid $2.5\cdot10^{-2}\,M$. 10 mg protein as an aqueous extract of acetone dried washed rat liver residue[2,6], and 2 mg muscle preparation[2] were added per tube. Final volume, 2.0 ml. Incubation at 38° for 20 minutes.

Glutamate derivative added *μM*	*Intermediate formed** *μM*	*Glutamate derivative added* *μM*	*Intermediate formed** *μM*
none	0.20	0.06 CG	0.25
0.05 AG	1.14	0.10 CG	0.31
0.10 AG	2.06	0.40 CG	0.66
0.20 AG	4.20	1.00 CG	1.38
0.40 AG	7.00	2.00 CG	2.40
1.00 AG	10.50	3.00 CG	3.42
2.00 AG	11.32	10.00 CG	9.32
3.00 AG	11.62		

* Estimated as citrulline[2].

The experiments of Table II show that up to 20 times as much Compound X can be obtained as there is AG present, whereas the activity of CG is apparently stoichiometric. However, correction for the amount of spontaneous decomposition of Compound X during incubation would give values approximately 25% higher than those shown in Table II. K_M values calculated from LINEWEAVER AND BURK[8] plots by the method of least squares are $2.0 \cdot 10^{-4}$ M for AG and $3.7 \cdot 10^{-3}$ M for CG. The data clearly show that with the experimental conditions employed in the present studies there is not a stoichiometric relation between glutamate derivative added and Compound X formed as had previously been reported[2,3].

Compound X isolated from incubation mixtures containing either CG or AG is converted to citrulline by a purified liver enzyme system[2] and by bacterial extracts at the same rate as synthetic carbamyl phosphate. Experiments with the carbamyl aspartic acid synthesizing system from rat liver[9] by Dr. J. M. LOWENSTEIN reveal that carbamyl phosphate can replace Compound X. Further, both synthetic carbamyl phosphate and isolated Compound X are converted to carbon dioxide, ammonia and ATP at the same rate by extracts of *S. faecalis* (V. A. KNIVETT and R. O. MARSHALL, unpublished experiments).

Analysis, by the method of KORITZ AND COHEN[10], of the isolated Compound X formed in the presence of CG apparently indicated that one mole of CG was present per mole of Compound X[3,6]. To our surprise similar results were obtained when the method was applied to the intermediate formed in the presence of AG. Further information was sought by setting up a system which simulated a compound X incubation mixture after synthesis of the intermediate had occurred. This system was supplemented with synthetic carbamyl phosphate and immediately deproteinized. Reisolation of carbamyl phosphate from this mixture showed it to fractionate in the same manner as Compound X[3,6]. Though synthetic dilithium carbamyl phosphate was not chromogenic by the method of KORITZ AND COHEN the reisolated cyclohexylammonium carbamyl phosphate was now chromogenic. It must be concluded that the color produced by this method when applied to cyclohexylammonium salts isolated from the incubation mixtures is in some manner an artifact of isolation.

These data are consistent with the concept that carbamyl phosphate is the intermediate carbamyl donor in enzymic citrulline synthesis by both mammalian liver and bacterial enzymes. While the outstanding contribution of JONES *et al.*[7] has clearly established this from their experiments, it is now clear that conditions for the synthesis and actual isolation of carbamyl phosphate using soluble liver enzyme systems were achieved in earlier studies from this laboratory[2,3,6], though it was erroneously thought to be a glutamate containing compound. In contrast to the bacterial system, the data of Table II clearly show that the animal system requires catalytic amounts of a glutamate derivative for optimum synthesis of carbamyl phosphate. The nature of this catalytic role is at present under investigation.

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