probable that one oxygen of the CO_2 (or H_2CO_3) as formed enzymically is derived from the ureido group of the citrulline and one from the orthophosphate.

TABLE I

ENZYMATIC FORMATION OF CO_2 IN PRESENCE OF O^{18}-LABELED ORTHOPHOSPHATE

A 250-ml. round-bottom flask contained, in 0.2 *M* succinate buffer, 60 micromoles of L-citrulline, 100 micromoles of $MgSO_4$, 50 micromoles of adenylic acid, and 2.5 millimoles of potassium phosphate in a total volume of 12.5 ml. at *p*H 5.3. A 50-ml. round-bottom flask contained 5 ml. of a *Pseudomonas* extract prepared according to Slade, *et al.*[3] The flasks were arranged so that they could be attached to a high-vacuum system and the contents intermixed. The contents of both flasks were frozen in Dry Ice and acetone, the flasks evacuated to a pressure of <0.1 micron, isolated from the vacuum source and thawed; this process was repeated three times to removed dissolved gases. The contents of the flasks were then brought to 37°, mixed, and incubated in the 250-ml. flask for 15 minutes with constant swirling. The flasks were then immersed in Dry Ice and acetone for 1 hour, the CO_2 collected with aid of a Toepler pump, and mass 46/44 ratio determined. The CO_2 output as measured in a Warburg apparatus under the same conditions but with 1/10 the amount of all materials was 3.4 micromoles in 20 minutes. For the urease experiments conditions were similar except that the L-citrulline was replaced by 10% urea and the *Pseudomonas* preparation replaced by an amount of a jack bean meal urease suspension sufficient to give approximately the same amount of CO_2 as obtained with the bacterial preparation and citrulline.

Enzyme	Substrate	Original atom % excess O^{18} in ortho-phosphate	Mass 46/44 ratio of CO_2[a]	Atom % excess O^{18} in CO_2
Pseudomonas/ extract	L-citrulline	0	0.00413[b]	0.00
Pseudomonas/ extract	L-citrulline	0.382[c]	.00518	.054
Urease	Urea	0	.00411	.00
Urease	Urea	0.392	.00412	.00

[a] Mass spectrometer analyses were made through cooperation of Dr. A. O. C. Nier and Mr. B. Donnally. [b] Mass 46/44 ratio for a control sample of tank CO_2 was 0.00413. [c] Orthophosphate isolated from the reaction mixture after incubation contained 0.354 atom % excess O^{18}.

The control experiments with urease reported in Table I demonstrate the absence of non-enzymic exchange of the oxygen of CO_2 and phosphate and that the oxygen from phosphate does not appear in the CO_2 produced by an enzyme reaction not involving uptake of orthophosphate.

Any mechanism suggested for the enzymic formation of ATP coupled with CO_2 formation from citrulline must account for the appearance of oxygen from orthophosphate into the CO_2. The simplest explanation is as suggested earlier, *i.e.*, the intermediate formation of an anhydride of free or of a substituted carbonic acid and orthophosphoric acid, and subsequent transfer of phosphate directly or eventually to ADP with cleavage of the O–P bond of the C–O–P linkage. Alternatively, an oxygen of CO_2 could come from another oxygen containing intermediate which in turn obtained an oxygen from orthophosphate. For example, transfer of an oxygen from one carboxy acid $RCOO^-$, to the acyl moiety of R'CO-S coenzyme A, to form $R'COO^-$ has been demonstrated in the coenzyme A transferase reaction.[7] Results and suggestions as given herein may apply to other related CO_2 fixation and release reactions.

(7) A. B. Falcone and P. D. Boyer, unpublished experiments.

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ENZYMATIC FORMATION OF FORMYLASPARTIC ACID FROM IMIDAZOLEACETIC ACID[1]

Sir:

Although the formation of β-[imidazolyl-4(5)]-acetic acid (ImAA) from histamine has been demonstrated in both mammalian and microbiological systems,[2] very little is known about its further metabolism. We are now reporting the isolation of formylaspartic acid as a product of the degradation of ImAA by a partially purified enzyme from an ImAA-adapted *Pseudomonas* sp. strain #6[3]; this reaction requires both reduced diphosphopyridine nucleotide (DPNH) and oxygen.

Except that ImAA[4] was used as a major carbon and nitrogen source, cells were grown and extracts were made as previously described.[5] The activity of the enzyme was assayed by measuring the rate of oxidation of DPNH ($-\Delta$O.D. $_{340\ m\mu}$); one μM. of DPNH was oxidized per μM. of ImAA consumed. The enzyme was purified about 200-fold and appears to be relatively specific for ImAA, since the rate of oxidation of DPNH with imidazolepropionic acid was less than 5% of that with ImAA and no reaction was observed with histamine, imidazole, and imidazole-lactic acid.

TABLE I

Assay	Experimental Δμ M.	Control Δμ M.
ImAA	−10.0	0
Acid-labile aspartic acid	+ 9.4	0
Ammonia	+ 9.8	0
Oxygen consumption	− 9.6	−0.14
Carbon dioxide production	+ 0.6	+0.4

The incubation mixture (1.2 ml.) contained 10 μM. of ImAA, 100 μM. of tris-hydroxymethylaminomethane buffer (*p*H 9.4), 200 μM. of glucose, 120 units of glucose dehydrogenase (H. J. Strecker and S. Korkes, *J. Biol. Chem.*, 196, 769 (1952)), 0.1 μM. of DPN and 0.5 ml. of enzyme. The control incubation mixture was the same except for the omission of glucose dehydrogenase and glucose; incubation 70 min. at 30°.

ImAA was assayed by coupling with diazotized 4-nitroaniline[2a]; ammonia was determined by Nesslerization after alkalinization with sodium carbonate and diffusion in Conway dishes. Acid-labile aspartic acid was determined after hydrolysis in 1 *N* H_2SO_4 (100°, 30 minutes); no aspartic acid was detectable before hydrolysis. L-Aspartic acid determinations were carried out by the *Clostridium welchii* decarboxylase method (A. Meister, H. A. Sober and S. V. Tice, *J. Biol. Chem.*, 189, 577, 591 (1951)); D-aspartic acid does not react in this assay.

(1) This investigation was supported in part by a research grant (G 3727) from the National Institutes of Health.

(2) (a) A. H. Mehler, H. Tabor and H. Bauer, *J. Biol. Chem.*, **197**, 475 (1952); (b) H. Tabor, A. H. Mehler and R. W. Schayer, *ibid.*, **200**, 605 (1953); (c) L. P. Bouthillier and M. Goldner, *Arch. Biochem.*, **44**, 251 (1953); (d) K. Satake, S. Ando and H. Fujita, *J. Biochem.*, **40**, 299 (1953).

(3) R. Y. Stanier and O. Hayaishi, *Science*, **114**, 326 (1951).

(4) Prepared in collaboration with Dr. Hugo Bauer.

(5) O. Hayaishi, THIS JOURNAL, **75**, 4367 (1953).

This preparation carried out the over-all reaction

$$\text{ImAA} + \text{DPNH} + \text{H}^+ + \text{O}_2 + \text{H}_2\text{O} \longrightarrow \text{Formylaspartic acid} + \text{DPN} + \text{NH}_3$$

Data indicating the stoichiometry are shown in Table I for an experiment in which DPNH was generated *in situ* from DPN by glucose plus glucose dehydrogenase. Formylaspartic acid was isolated and identified by *melting point:* 136–137° (authentic sample 135–137°; mixed m.p. 135–137°), *analysis*[5] ((isolated material): *calculated* for $C_5H_7O_5N$: C, 37.27; H, 4.37; N, 8.70. *Found:* C, 37.36; H, 4.73; N, 8.67) and the *infra-red spectrum.*[6]

The mechanism of the intermediate steps in this over-all reaction is still unclear. The obligatory requirement for both DPNH and oxygen may possibly be explained either by the unfavorable equilibrium of the primary reaction or by a peroxidative step utilizing peroxide formed by the enzymatic oxidation of DPNH. The latter possibility was rendered less likely by the absence of DPNH oxidase in the purified preparation; furthermore no ImAA was utilized when ImAA was incubated with the enzyme in the absence of DPNH, even when hydrogen peroxide was added or generated by the action of glucose oxidase [notatin] on glucose. Further purification of the enzymes involved appears to be necessary to elucidate the nature of the various steps.

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(6) The elementary analyses were carried out by the Microanalytical Laboratory of the N. I. H. under the direction of Dr. W. Alford; the infrared spectra were obtained by Mr. H. K. Miller and Mrs. P. B. Humphries of this institute.

(7) With the technical assistance of Natalie Ann Fraser.

THE FORMATION OF CARBAMYL ASPARTIC ACID BY RAT LIVER PREPARATIONS[1]

Sir:

Carbamylaspartic acid (CA) has been shown to be a precursor of pyrimidines.[2-6] We have obtained evidence that CA is formed by the enzymatic transfer of a carbamyl group ($-CO\cdot NH_2$) from compound X[7] to aspartic acid.

Preliminary evidence of this reaction was obtained by incubating aspartic acid, acetylglutamic acid, bicarbonate, ammonium chloride, phosphate buffer, and ATP with rat liver acetone powder extracts. CA was one of the products of the reaction using whole rat liver acetone powder. It was not formed when rat liver particulate fraction or supernate acetone powder extracts were used alone.

A solution of C^{14} labeled acetyl-compound X was prepared using acetylglutamic acid and $C^{14}O_2$ by a method which followed that described previously for the preparation of carbamyl-compound X from carbamylglutamic acid.[8] An alcohol fraction (final concentration 40%) of rat liver particulate acetone powder was used as source of enzyme. The solution was deproteinized with $HClO_4$ (during this treatment unreacted $C^{14}O_2$ was removed) and neutralized to *p*H 7.2 with sodium bicarbonate; 6.4 μ-moles of labeled acetyl-compound X was incubated with acetone powder or lyophilized powder extracts of rat liver supernate (48 and 104 mg. protein, respectively) in the presence or absence of 100 μ-moles aspartic acid. (Compared on a protein basis, the lyophilized powder possessed about four times the activity of the acetone powder.) After deproteinization, the solutions were chromatographed on Dowex-2 anion exchange resin (formate form). The fractions were assayed for radioactivity. The compound from the elution peak which coincided with that of authentic CA was isolated by freeze drying the pooled fractions and removing the buffer salt by sublimation. This peak was absent when aspartic acid was omitted from the incubation. A portion of the compound was identified by isotope dilution with authentic CA (D, L-form) and conversion to hydantoin-5-acetic acid.[9] The melting point remained constant (174°) and the specific activity was 14,000, 13,370, 13,710 and 13,230 c.p.m. per μ-mole after each of four recrystallizations as CA. The melting point remained constant (212°) and the specific activity was 13,490 and 13,490 c.p.m. per μ-mole after each of two recrystallizations as hydantoin-5-acetic acid.

The isolated compound was assayed for radioactivity and CA.[10] Its specific activity compared with that of labeled acetyl-compound X was: CA, 3.25×10^5 c.p.m. per μ-mole; compound X, 3.49×10^5 c.p.m. per μ-mole. (Specific activity of labeled acetyl-compound X was determined on the basis of radioactivity assays and enzymatic assay for compound X.) It is unlikely that the CA was synthesized *de novo* by a pathway not involving compound X, since the specific activity of unreacted $C^{14}O_2$ remaining after the acidification step in the preparation of compound X was greatly reduced by neutralizing the reaction mixture with $NaHCO_3$.

ADDENDUM.—After the present communication was submitted, two papers bearing on our findings appeared. L. H. Smith and D. Stetten (THIS JOURNAL, **76**, 3864 (1954)) reported that C^{14} labeled citrulline was a precursor of orotic acid in rat liver slices. This finding was interpreted as evidence for the formation of CA as an intermediate. P. Reichard (*Acta Chem. Scand.*, **8**, 795 (1954)) reported that mitochondrial preparations from rat liver were capable of synthesizing CA from aspartic

(1) This investigation was supported in part by a research grant A-540 (C3) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service, and the Wisconsin Alumni Research Foundation.

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(10) S. B. Koritz and P. P. Cohen, *J. Biol. Chem.*, **209**, 145 (1954).