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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acid, ammonia and carbon dioxide provided that glutamate or carbamylglutamate was present.

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THE DIRECT FORMATION OF ACETYL-COENZYME A FROM SUCCINATE[1]

Sir:

Although extracts of the ciliated protozoan, *Tetrahymena*, reduce cytochrome c in the presence of succinate,[2] the low cytochrome oxidase activity of the extracts[3] indicates that the main route of succinate utilization is not through the usual succinic dehydrogenase–cytochrome system path. Recent experiments with ciliate extracts have revealed that in the presence of succinate, ATP[4] and Co A, there occurs a rapid formation of acetyl-Co A, as measured by the hydroxamate method.[5]

Washed suspensions[6] of *Tetrahymena pyriformis*, strain S, were homogenized by five passages through a Logeman hand mill. After centrifugation for 20 min. at 1350 × *g* the precipitate was suspended in 1 *M* tris buffer *p*H 8.4. Most of the succinic dehydrogenase activity, as measured by the reduction of cytochrome c[2], is in the supernatant. A slight but measureable activity is retained in the precipitate. In the presence of ATP, Co A, Mg^{++} and succinate, the precipitate forms a hydroxamic acid. Table I shows that there is no significant amount of hydroxamate formation in the absence of these components.

On first examination the reaction seems to be similar to the decarboxylation of succinate where succinyl Co A and propionyl Co A are intermediates.[7] However, the results obtained upon extraction of the formed hydroxamate and chromatography on paper with water saturated butanol as solvent[8] excludes this possibility. The developed chromatograms show only a single spot at R_F 0.51 which is identical to that of acetyl hydroxamate prepared from acetyl phosphate. Mixtures of the extracted hydroxamate and acetyl phosphate also yield a single spot. In addition, no significant amounts of carbon dioxide are formed when the enzyme is incubated under nitrogen with the components listed in Table I. No hydroxamate is recovered when hydroxylamine is added to the mixture (containing 100 μM. of potassium phosphate buffer in place of hydroxylamine) at the end of the incubation period; this appears to preclude the formation of an intermediate acyl phosphate.

Since the extract contains very low succinic dehydrogenase activity, and, as would thus be anticipated, malonate does not inhibit the reaction (Table I), acetyl formation is not achieved through Krebs cycle oxidation of the succinate. Formation of the acetyl group by reversal of the cycle, through α-ketoglutarate, also seems unlikely, since no trace of a succinyl hydroxamate or phosphate is obtained on the paper chromatograms.

TABLE I

Components	μM. hydroxamate formed
Complete	2.23
No succinate	0.64
No ATP	0.30
No Co A	0.06
No Mg^{++}	0.87
Complete + 100 μM. malonate	2.73

The complete mixture contained in 1.0 ml.: 200 μM. Na succinate, 25 units Co A, 10 μM. K-ATP, 50 μM. NaF, 15 μM. $MgCl_2$, 400 μM. hydroxylamine, 20 μM. glutathione, 100 μM. tris buffer *p*H 8.4, and extract containing 22 mg. of protein.

In the absence of Co A, succinate and the protozoan enzyme do not replace acetate in the formation of acetyl phosphate by extracts of *Streptococcus faecalis* which contain acetokinase activity.[9] Acetyl formation is thus not the combination of a C_2–C_2 split to free acetate and acetate activation to form acetyl-Co A.

Equimolar amounts of inorganic phosphate and acetyl groups are formed by the reaction. The ratio is not affected by fluoride.

Succinic acid is the only carboxylic acid revealed when ether extracts of the reaction products are chromatographed on paper with *n*-butanol–formic acid–water (5:1:4) as the solvent system.[10]

These observations indicate the direct formation of acetyl-Co A from succinate by extracts of *Tetrahymena*.

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(4) The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; Co A, coenzyme A; tris, tris-(hydroxymethyl)-aminomethane; Pi, inorganic phosphate.

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THE ENZYMATIC FORMATION OF RIBULOSE DIPHOSPHATE

Sir:

We have recently reported that a soluble extract from spinach leaves is capable of fixing carbon dioxide in the carbonyl group of phosphoglyceric acid in the absence of light.[1] Ribose 5-phosphate, TPN, ATP, and Mg^{++} are required in this reaction. Fractionation of this crude extract has now yielded a preparation which, in the absence of TPN, catalyzes the formation of ribulose diphosphate (RuDP) from ATP and ribose 5-phosphate. This reaction requires Mg^{++}. The activity has been purified about ten-fold by ammonium sulfate precipitation and adsorption and elution on calcium phosphate gel.

To isolate the product formed in the reaction, ATP labeled with P^{32} in the two terminal phosphate groups was incubated with ribose 5-phosphate and Mg^{++} in the presence of the partially purified enzyme. Ion exchange chromatography of the

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