markedly the activity of the washed sediment but not of the crude preparations (Table II).

TABLE II

Preparation	Additions	Initial rates of Pi uptake in the light μM. P/hr./ mg. protein	μM. P/hr./unit opt. d. at 800 mμ
Crude sonic		2.0	22.6
	DPN	1.9	21.5
	DPN, KGA	1.9	21.5
		2.1	9.1
Resuspended	DPN	2.1	9.1
sediment	KGA	4.3	18.7
	DPN, KGA	5.1	22.1
	DPN, KGA, cyt. c	5.1	22.1
	TPN, KGA	4.4	19.1

All preparations contained the following additions per ml.: 12 μM. $MgCl_2$, 10 μM. KF, 7 μM. P_i, 10.3 μM. ADP. Other additions were present as indicated in the following concentration per ml.: 0.5 μM. DPN, 0.25 μM. TPN, 0.012 μM. cyt. c, 15 μM. KGA. Resuspended sediment consisted of top half of sediment obtained after centrifugation of crude sonic preparation for 1 hour at 135,000 × gravity. Other experimental conditions the same as indicated for Table I.

Light dependent phosphorylation of ADP did not require oxygen and was not affected by 10^{-4} *M* dinitrophenol or 10^{-3} *M* iodoacetamide, 10^{-3} *M* hydrogen cyanide and 10^{-3} *M* mersalyl[4] gave inhibitions between 25–50%, 10^{-3} *M* *o*-phenanthroline inhibited completely, and 2,6-dichlorophenolindophenol inhibited phosphorylation from 60 to 100%. The system was completely inactivated after incubation at 80° for two minutes. Slow freezing and thawing of the preparation also caused extensive inactivation. Preparations kept on ice in the dark for eight hours did not show an appreciable loss of activity.

Similar systems have been described in the case of chloroplast preparations from higher plants.[6,7] In these systems, however, labelled P_i was employed to demonstrate the light induced formation of ATP.

I wish to thank Dr. F. Lipmann for his hospitality and advice during this investigation, and Drs. A. Brodie, M. E. Jones, R. Bandurski and J. A. Johnston for their many helpful suggestions.

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(7) D. I. Arnon, M. B. Allen and F. R. Whatley, *Nature*, **174**, 394 (1954).

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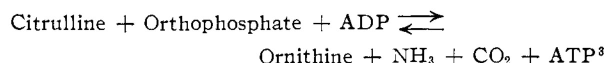
INCORPORATION OF PHOSPHATE OXYGEN INTO CARBON DIOXIDE FORMED BY ENZYMIC DEGRADATION OF CITRULLINE COUPLED WITH ATP SYNTHESIS[1]

Sir:

The biosynthesis of citrulline from ornithine by animal tissues requires adenosine triphosphate (ATP)[2] while the bacterial degradation of the ureido group of citrulline is coupled to ATP synthesis according to the over-all equation



$$\text{Citrulline} + \text{Orthophosphate} + \text{ADP} \rightleftharpoons \text{Ornithine} + NH_3 + CO_2 + \text{ATP}^{3}$$

From knowledge of the fate of phosphate oxygen in the enzymic formation of 3-phosphoglycerate and in phosphate transfer reactions,[4] the intermediate formation of an anhydride compound between carbonic acid or a derivative thereof and orthophosphoric acid accompanying CO_2 release and ATP synthesis would be expected to result in incorporation of phosphate oxygen into the CO_2.

It appeared feasible to check this possibility with the use of bacterial preparations[5] which catalyze the above reaction provided that the exchange of oxygen of carbon dioxide with that of water could be sufficiently minimized to allow detection of excess O^{18} from O^{18}-labeled orthophosphate in the CO_2 formed.

Using conditions similar to those described in Table I, a small but definite incorporation of excess O^{18} of orthophosphate into the CO_2 formed was found (mass 46/44 ratio of CO_2 = 0.00431). Increased yield of O^{18} in the CO_2 was obtained with change of conditions to reduce the exchange of oxygen of CO_2 with that of water before escape of CO_2 into the evacuated reaction chamber; *i.e.*, decrease in total liquid volume from 30 to 17.5 ml., increase in flask size from 100 to 250 ml. and with the reaction mixture strongly swirled instead of occasionally mixed. Results of such experiments are shown in Table I; they give conclusive proof that oxygen from orthophosphate appears in the CO_2 formed. The CO_2 produced from citrulline in presence of unlabeled orthophosphate contained, within experimental error, the same amount of O^{18} as tank CO_2. The amount of O^{18} in the CO_2 from the sample containing orthophosphate-O^{18} was approximately 28% of that expected if one oxygen of phosphate appeared in the CO_2 formed directly as a gas or 43% of that expected if one oxygen of phosphate appeared in H_2CO_3 which liberated gaseous CO_2. Under the reaction conditions at 37° with phosphate present (without consideration of any possible carbonic anhydrase activity of the bacterial preparation), the half-time for complete exchange of CO_2 oxygen with that of water was estimated from the data of Mills and Urey[6] to be about 19 seconds. That relatively rapid exchange was occurring was also shown by the increased yield of O^{18} in the CO_2 obtained as a result of reduction in liquid volume, increase of size of reaction flask, and vigorous swirling of the reaction mixture. These considerations together with the known specificity of enzyme reactions make it

(1) Supported by a grant from the National Science Foundation and grant 1033 from the National Institutes of Health, Public Health Service. Contribution No. 3226, Scientific Journal Series, Minnesota Agricultural Experiment Station.

(2) S. Grisolia and P. P. Cohen, *J. Biol. Chem.*, **204**, 753 (1953); **198**, 561 (1952).

(3) H. D. Slade, C. C. Doughty and W. C. Slamp, *Arch. Biochem. Biophys.*, **48**, 338 (1954); V. A. Knivett, *Biochem. J.*, **56**, 602 (1954); M. Korzenovsky and C. H. Werkman, *ibid.*, **57**, 343 (1954).

(4) P. D. Boyer and W. H. Harrison in W. D. McElroy and B. Glass, "Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Md., p. 658 (1954); M. Cohn, *J. Biol. Chem.*, **201**, 735 (1953).

(5) We are indebted to Dr. H. D. Slade for kindly supplying us with a culture of the Pseudomonas used in his studies.

(6) G. A. Mills and H. C. Urey, THIS JOURNAL, **62**, 1019 (1940).

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 ΔμM.	Control ΔμM.
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.

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

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