Bicarbonate-dependent enzymic phosphorylation of fluoride by adenosine triphosphate*

The pathway of propionate metabolism in animal tissues has been shown[1,2] to involve (a) activation to propionyl CoA**, (b) an ATP-dependent carboxylation of propionyl CoA to methylmalonyl CoA, and (c) an isomerization of methylmalonyl CoA to succinyl CoA or succinate, followed by oxidation of succinate via the citric acid cycle. Thus, phosphate bond energy is required for the carboxylation reaction, but apparently not for the isomerization[3]. In the course of an investigation of the fate of ATP in the carboxylation step and of the mechanism by which its energy is made available for the reaction, a bicarbonate- and fluoride-requiring enzymic cleavage of ATP has been discovered, which yields ADP and monofluorophosphate ($PO_3F^=$) as products. The implications of this reaction for a mechanism of enzymic activation of carbon dioxide will be discussed.

With a propionyl CoA carboxylase enzyme system purified 10-fold from pig heart extracts and free of demonstrable inorganic pyrophosphatase activity, a liberation of orthophosphate from ATP in excess of that caused by ATPase (Table I, experiment 7) is obtained on addition of both propionyl CoA and bicarbonate-CO_2 but not of either alone (Table I, experiments 1, 3, and 5). Orthophosphate was determined colorimetrically[4]. As further shown in Table I, the requirement for $KHCO_3$ is not due to the potassium ion, nor to an effect on pH. Propionyl CoA cannot be replaced by CoA. Since the addition of an excess of crystalline pyrophosphatase*** (PPase) had no effect on the amounts of orthophosphate liberated (Table I), it was concluded that orthophosphate, and not pyrophosphate, is released from ATP in the carboxylase reaction.

TABLE I

ORTHOPHOSPHATE LIBERATION FROM ATP IN PROPIONYL CoA CARBOXYLATION REACTION

No.	*System*	*Other additions*	*pH at end of incubation*	*Orthophosphate liberated* (μmoles)*
1	Complete		7.32	0.62
2	Complete	PPase	7.33	0.64
3	No $KHCO_3$, no CO_2		7.28	0.33
4	No $KHCO_3$, no CO_2	PPase	7.15	0.31
5	No propionyl CoA		7.32	0.14
6	No propionyl CoA	PPase	7.38	0.17
7	No propionyl CoA, no $KHCO_3$, no CO_2	PPase	7.23	0.27
8	No propionyl CoA	CoA, PPase	7.26	0.17
9	No $KHCO_3$, no CO_2	KCl, PPase	7.23	0.24

The complete system contained the following components (in μmoles), Imidazole buffer, pH 7.3, 100; reduced glutathione, 5; $MgCl_2$, 4; ATP (cryst.), 4; $KHCO_3$, 380; propionyl CoA, 2; enzyme (acetone fraction), 1.0 mg. Final volume, 1.5 ml. Gas phase, 100% CO_2. Incubation in Warburg flaks, 40 min at 30°. Other additions: PPase, 0.02 mg; CoA, 1.0 μmole; KCl, 380 μmoles. CO_2-free solutions and air used when $KHCO_3$ was omitted.

* Results from several experiments.

Because of COON'S[5] report that pig heart extracts catalyzed a bicarbonate-dependent cleavage of ATP to AMP and pyrophosphate, the above experiments were repeated in the presence of fluoride to inhibit any traces of pyrophosphatase which might contaminate our carboxylase preparations. After complete removal of nucleotides by charcoal treatment[6] of the protein-free supernatant, the amounts of orthophosphate present were comparable to those formed in the

* Aided by grants from the National Institute of Arthritis and Metabolic Diseases (Grant A-529) of the National Institutes of Health, United States Public Health Service; the American Cancer Society (recommended by the Committee on Growth National Research Council); the Rockefeller Foundation; and by a contract (N6onr279-T.O.6) between the Office of Naval Research and New York University College of Medicine.

** Abbreviations: Adenosine-5'-monophosphate, -diphosphate, and -triphosphate, AMP, ADP, and ATP, respectively; coenzyme A, CoA.

*** We are indebted to Dr. M. KUNITZ for a gift of this enzyme.

absence of fluoride. Unexpectedly, however, a very rapid decomposition of ATP was now found to have occurred, as indicated by the presence of large amounts of a non-nucleotide phosphate compound (XP) which released all of its phosphate as orthophosphate on hydrolysis for 10 minutes in 1.0*N* HCl at 100°. That XP was not inorganic pyrophosphate was soon evident from, among other things, its rate of migration as a single component on paper chromatograms, as measured both with a molybdic acid spray reagent[7] and autoradiographically in experiments with ^{32}P-labelled ATP*.

An incubation in the presence of $^{14}CO_2$ revealed that XP did not contain carbon dioxide. By paper chromatography it was possible to eliminate, in addition to ortho- and pyrophosphate, tripolyphosphate, trimetaphosphate, and other "metaphosphates". Analysis of an eluted crude sample of XP revealed the absence of Mg^{++} and the presence of about equimolar amounts of fluoride and phosphorus, amounting to 4.6 and 5.9%, respectively**. A sample of monofluorophosphate was accordingly synthesized[8] and purified as the disilver salt[9]. On chromatography it showed the same R_F values and characteristic slow color development as XP, in two solvent systems***. A comparison of hydrolysis rates provided further evidence that XP is fluorophosphate. Thus, the first order velocity constants of hydrolysis of pyrophosphate, fluorophosphate, and XP. were 2.7, 67 and 63 ($\times 10^{-5}$ sec^{-1}), respectively, in 0.5*N* HCl at 40°, and 2, 450, and 410 ($\times 10^{-7}$ sec^{-1}) in 2.0*N* NaoH at 100°.

As shown in Table II, the formation of fluorophosphate depends completely on the presence of enzyme, Mg^{++}, bicarbonate-CO_2, ATP, and fluoride. ATP cannot be replaced by ortho- or pyrophosphate. The stoichiometric formation of ADP and fluorophosphate from ATP is shown in Table III. The small amount of reaction occurring without added bicarbonate-CO_2 (Experiment 2) may be attributed to the presence of some bicarbonate in the reagents.

The "fluorokinase" activity can be readily separated by fractionation from at least one protein component of the propionyl CoA carboxylase system. Evidence that the kinase is an essential component of the carboxylase system rests so far only on the observation that it has been concentrated in the purified carboxylase preparation and on the fact that methylmalonate synthesis is decreased in the presence of fluoride and fluorophosphate synthesis is diminished in the presence of propionyl CoA.

TABLE II

REQUIREMENTS FOR ENZYMIC FORMATION OF FLUOROPHOSPHATE

System	*Other additions (μmoles)*	*Fluorophosphate formed (μmoles)*
Complete		1.86
Complete but with 150 μmoles of KF		3.27
No enzyme		0
No $MgCl_2$		0.08
No KF (^{32}P-ATP)		0**
No $KHCO_3$, no CO_2	Imidazole buffer, pH 7.3, 100; KCl, 380	0.06
With 20 μmoles of $KHCO_3$, no CO_2 (gas phase, air)	Imidazole buffer, pH 7.3, 100	0.18
Complete	Imidazole buffer, pH 7.3, 100	1.8
No ATP	^{32}P-pyrophosphate, 1; ^{32}P-orthophosphate, 0.4	0**

The complete system contained the following components (in μmoles), $MgCl_2$, 4; ATP, 4; $KHCO_3$, 380; KF, 30; enzyme ($(NH_4)_2SO_4$ or acetone fraction), 1.0 mg pH 7.3 with 100% CO_2 in gas phase. Incubation, 30 min at 30°.

* Determined as orthophosphate released by hydrolysis in 1.0 *N* HCl at 100° for 10 min after removal of nucleotides with charcoal. Results from several experiments.

** Assayed by autoradiography of paper chromatograms.

* Prepared by the Schwarz Laboratories, Mount Vernon, N.Y.

** Analyses by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

*** Solvent 1, *iso*propanol (90 ml), 90% formic acid (80 ml), concentrated NH_4OH (1.0 ml); Solvent 2[10], *iso*propanol (75 ml), water (25 ml), trichloroacetic acid (5 g), concentrated NH_4OH (0.3 ml).

TABLE III

STOICHIOMETRY OF FLUOROPHOSPHATE-FORMING REACTION

Changes per ml reaction mixture; values expressed in μmoles.

No.	Orthophosphate	Fluorophosphate	Total inorganic phosphate (found)	ATP	ADP	AMP	Total inorganic phosphate (calc.)
1	+0.25	+2.31	+2.56	−2.17	+2.07	+0.10	+2.27
2	+0.22	+0.42	+0.64	−0.58	+0.58	0	+0.58
3	+0.25	+2.10	+2.35	−2.08	+1.98	+0.11	+2.20
4	+0.25	+2.06	+2.31	−2.08	+1.97	+0.11	+2.19

Conditions: 4 μmoles each of $MgCl_2$ and ATP in a final volume of 1.5 ml. Incubation, 40 min at 30°. In addition, experiment 1, KF (150 μmoles), $KHCO_3$ (380 μmoles), gas, CO_2, enzyme (1 mg of acetone fraction); experiment 2, KF (150 μmoles) *no added* $KHCO_3$, gas, N_2, imidazole buffer (100 μmoles), pH, 7.0, enzyme (1.0 mg); experiment 3, KF (300 μmoles), $KHCO_3$ (380 μmoles), gas, CO_2, enzyme (1.5 mg); experiment 4, KF (150 μmoles), $KHCO_3$ (380 μmoles), gas, CO_2, enzyme incubated with 0.001 *M* N-ethylmaleimide for 30 min at room temp. and dialyzed. Orthophosphate and fluorophosphate determined after removal of nucleotides with charcoal; nucleotides determined by ultraviolet absorption after paper chromatography as described by KREBS AND HEMS[11].

Since orthophosphate does not itself give rise to fluorophosphate in the "fluorokinase" reaction, it cannot be a primary product of the cleavage of ATP. The requirement for bicarbonate suggests to us that "fluorokinase" may actually be a carbonokinase, catalyzing the formation, from CO_2 and ATP, of carbonylphosphate, possibly bound in some form preventing its hydrolysis. Carbonylphosphate might then react either with propionyl CoA, in the presence of propionyl CoA carboxylase, yielding methylmalonyl CoA and orthophosphate, or (perhaps non-enzymically) with fluoride, yielding fluorophosphate and CO_2. Experiments are in progress to test this hypothesis.

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Received March 20th, 1956

* Postdoctoral fellow of the American Heart Association.
** Doherty Foundation fellow.