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The Formation of Phosphopyruvate by Washed Suspensions of Sheep Kidney Particles

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It was observed in previous experiments (Bartley, 1954) that washed, sheep-kidney particles synthesize several phosphate esters when incubated with either pyruvate, succinate or α -oxoglutarate, and ^{32}P -labelled phosphate. One of these compounds is identified in this paper as phosphopyruvate.

METHODS

Phosphopyruvate was prepared by the method of Ohlmeyer (1951). The other substances used, the analytical methods, and the preparation of the tissue suspensions were as previously described (Bartley, 1953; Bartley, 1954).

RESULTS

In the previous experiments (Bartley, 1954), the oxidation of α -oxoglutarate or succinate by suspensions of sheep-kidney mitochondria resulted in the formation of a phosphorylated compound which ran ahead of phosphate on the paper chromatogram when *p*-toluenesulphonic acid:*tert*.-pentanol:water was the solvent ('spot 1'). This substance rapidly became radioactive when the medium contained ^{32}P -labelled phosphate.

Identification of spot 1. On making a paper chromatogram with the above solvent, spot 1 had the same R_f as phosphopyruvate. This was the only phosphate compound of those tested (adenosine phosphates, hexose phosphates, glycerol phosphates, thiamine phosphates, pyrophosphate, pyridoxal phosphate) that had an R_f greater than that of phosphate. The addition of phosphopyruvate to a solution containing spot 1 gave no additional spot with any of the solvents tested (*tert*.-pentanol:formic acid; *p*-toluenesulphonic acid:*tert*.-pentanol:

water; isopropyl ether:formic acid). In common with phosphopyruvate, spot 1 gave no hydroxamic acid reaction and showed no ultraviolet absorption. The final identification of spot 1 was achieved by determining the phosphate and pyruvic acid content.

A sheep-kidney preparation (to which 0.0025M thiamine monophosphate was added) was incubated for 30 min. with 60 μmoles of sodium α -oxoglutarate and tracer amounts of ^{32}P -labelled phosphate. The reaction was stopped by the addition of 0.5 ml. of 30% trichloroacetic acid (TCA) and the supernatant solution was used for analysis. Approximately one-third of the total TCA extract (about 2.5 ml.) was put as a band on a 10 cm. line on each of three chromatogram papers, and 50 μl . marker spots were put 2 cm. away from each end of this line. The chromatograms were developed for 20 hr. in a *p*-toluene sulphonic acid:*tert*.-pentanol:water mixture and the papers were then dried at room temperature. The strips of paper bearing the marker spots were cut off and sprayed with molybdate (Hanes & Isherwood, 1949) to locate spot 1. The sprayed strips were laid beside the unsprayed chromatograms and the ends of the bands corresponding to spot 1 were traced. The location of the bands was confirmed and their areas delineated by plotting the areas of radioactivity associated with them. These areas were cut out, and the rest of the paper was sprayed with the molybdate reagent to confirm, from the appearance of the cutting line, that no other phosphorus-containing areas had been included in the excised paper. The material was eluted with water and made up to a volume of 2.3 ml. A 0.2 ml. sample showed no free phosphate when analysed by the method of Berenblum & Chain (1938). Within experimental error, the amounts of phosphorus liberated from 0.2 ml. samples by wet ashing, by hydrolysis for 2 hr. in 1N-HCl at 100°, or by treatment with alkaline iodine were identical (3.30, 3.40, 3.29 μg .), as were the specific activities of the phosphorus (3130, 3170 and 3030 counts/min./ μg . P). This strongly suggests that the material contained one phosphate compound only. Under these conditions phosphopyruvate is also completely hydrolysed.

Of especial significance is the liberation of phosphorus by alkaline iodine, which is considered specific for phosphopyruvate (Lohmann & Meyerhof, 1934). To identify pyruvic acid, a 1.0 ml. sample was heated for 2 hr. at 100° in a stoppered tube with 1.0 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl. A 0.4 ml. sample of this material was analysed for phosphate, and the remainder was shaken with successive 1.0 ml. portions of ethyl acetate to extract the phenylhydrazones. In the combined ethyl acetate extracts, pyruvic acid was determined by the paper ionophoretic method of Neish (1953). A 0.5 ml. sample of the original solution was treated in the same way, omitting the 2 hr. hydrolysis. A standard mixture of pyruvic, α -oxoglutaric and oxaloacetic hydrazones was separated on the same paper as the two test solutions. The ionophoretic separation showed that acid hydrolysis had liberated a substance forming a 2:4-dinitrophenylhydrazone identical with that given by pyruvic acid.

The amount of pyruvic acid liberated was 57% of that expected from the amount of phosphate liberated. A pure sample of phosphopyruvate gave 63% of the theoretical amount of pyruvic acid when treated in the same way.

Conditions of formation of phosphopyruvate. Phosphopyruvate was formed when cocarboxylase, thiamine monophosphate, or thiamine was incubated with a washed suspension of sheep-kidney mitochondria and α -oxoglutarate, succinate, or pyruvate plus bicarbonate. Table 1 shows the amounts formed with these substrates. Phosphopyruvate was not formed from pyruvate when the bicarbonate concentration was below 0.0075M. Thiamine phosphates had been added because the experiments were primarily designed to study the metabolism of these substances. Phosphopyruvate was also formed in the absence of thiamine phosphates. It did not accumulate when the substrate concentration was lowered from 0.0125 to 0.0025M.

Paper chromatography showed that phosphopyruvate was also formed when fumarate, malate and oxaloacetate were the substrates. The quantities were of the same order as in the presence of α -oxoglutarate, succinate, or pyruvate plus bicarbonate, as judged by the colour intensities of the chromatogram.

Incorporation of radioactivity into phosphopyruvate. The rate of incorporation of ^{32}P -labelled phosphate into phosphopyruvate, and the labile phosphate of adenosine triphosphate (ATP) plus adenosine diphosphate (ADP) is shown in Table 2. On short incubation (5–10 min.), the specific activity of phosphopyruvate was much lower than that of ATP and phosphate; but later (after 30–60 min.) the specific activity of all three compounds became equal. The low specific activity of phosphopyruvate at the early stages indicates that phosphopyruvate was not formed directly from phosphate. The data are in accordance with the assumption that ATP is the phosphate donor in the synthesis of phosphopyruvate. The final isotopic equilibrium between ATP, phosphopyruvate and phosphate indicates that the phosphopyruvate formed is being further metabolized; the amount accumulating is thus a measure of the rates of its formation and disappearance. The turnover of phosphopyruvate appears to be very rapid. The addition of 10 μ moles of phosphopyruvate to a system forming phosphopyruvate did not alter the amount of phosphopyruvate (3 μ moles) found after 30 min. incubation, and no phosphopyruvate could be detected after 10 min. incubation when 10 μ moles of phosphopyruvate were added to a kidney suspension which, owing to a low concentration of succinate, does not accumulate phosphopyruvate.

Table 1. *Formation of phosphopyruvate from various substrates in washed sheep-kidney particles*

(Suspension of kidney particles (about 70 mg. dry wt. and 4 ml.) with ATP (0.001M), MgCl_2 (0.001M) and orthophosphate containing $^{32}\text{PO}_4^{3-}$. 'Phosphopyruvate formed' refers to 4 ml. incubated material.)

Expt. no.	Substrate		Ortho-phosphate P added (μ moles)	Thiamine derivative added		Period of incubation (min.)	Phosphopyruvate formed (μ moles)
	Nature	Quantity (μ moles)		Nature	Quantity (μ moles)		
1	Pyruvate + NaHCO_3	50.0	30	Cocarboxylase	8.0	60	4.45
2	α -Oxoglutarate	57.6	18.2	Cocarboxylase	8.3	10 20 30	1.77 1.33 2.67
3	Succinate	65.6	19.2	Cocarboxylase	6.2	10 20 30	0.56 1.04 3.26
4	α -Oxoglutarate	55.7	23.8	Thiamine monophosphate	6.9	5 10 15 20 30 60	0.85 0.93 0.47 0.49 2.26 4.44
5	α -Oxoglutarate	39.0	20.5	Thiamine	3.6	35 65	1.10 2.20

Table 2. Comparison of specific activities of orthophosphate, labile phosphorus of ATP, and phosphopyruvate
(Same series of experiments as in Table 1.)

Expt. no.	Substrate		Incubation time (min.)	Specific activity (counts/min./μg. P)		
	Nature	Quantity (μmoles)		Ortho-phosphate	Labile P of ATP + ADP	Phospho-pyruvate
1	Pyruvate + NaHCO ₃	50.0	0	670	0	0
		50.0	60	437	408	440
2	α-Oxoglutarate	57.6	0	2088	0	0
			10	1630	1370	530
			20	1260	1050	880
			30	1335	1325	1350
3	Succinate	65.6	0	1655	0	0
			10	1220	780	650
			20	1150	960	1070
			30	1300	1330	1330
4	α-Oxoglutarate	55.7	0	1545	0	0
			5	1430	1080	440
			10	1340	1140	750
			15	1330	1140	750
			20	1320	1290	1020
			30	1330	1210	1050
			60	1270	1200	1270

Table 3. Formation of phosphate esters by washed kidney particles

(Initially virtually all phosphate was present as ATP, thiamine phosphates and inorganic phosphate. The amount of phosphate recovered in these three forms after incubation was deducted from the total initial phosphate and is recorded as 'phosphate ester formation'. Same series of experiments as in Table 1.)

Expt. no.	Substrate added	Time of incubation (min.)	Phosphate ester formation (μmoles)	Phosphate ester	Phosphate ester
				accounted for as phospho-pyruvate (μmoles)	unaccounted for (μmoles)
1	Pyruvate + NaHCO ₃	60	9.64	4.45	5.19
2	α-Oxoglutarate	30	11.37	2.67	8.67
3	Succinate	10	7.57	0.56	7.01
		20	7.95	1.04	6.91
		30	10.36	3.26	7.10
4	α-Oxoglutarate	30	4.50	2.26	2.24
		60	10.38	4.44	5.94
5	α-Oxoglutarate	65	3.90	2.20	1.70

Accumulation of phosphate esters other than phosphopyruvate. As seen in Table 3, phosphopyruvate does not account for all the phosphate that disappears when kidney mitochondria are incubated with α-oxoglutarate, succinate or pyruvate plus bicarbonate. In addition to the phosphopyruvate spot, two, and in some cases three other phosphate-containing spots appear on the paper chromatogram. These have not been identified. Their *R_F* values are 0.23, 0.3 and 0.5. The first is the spot 4 described previously (Bartley, 1954). The two others do not occur when the pyruvate plus bicarbonate is the substrate. They begin to appear after a lag period of about 20 min. In Expt. 2 (Table 3), the last spot (*R_F* = 0.5) contained about 4 μmoles phosphate after 1 hr., i.e. about the same as the

phosphopyruvate spot. The spot of *R_F* 0.5 reached isotopic equilibrium with the phosphate at about the same time as the phosphopyruvate.

DISCUSSION

The aerobic formation of phosphopyruvate by kidney preparations *in vitro* has been recorded before by Kalckar (1939) (see also Leloir & Muñoz, 1944), who found phosphopyruvate, together with various other organic phosphates, under conditions of incubation similar to those used in the present experiments. Kalckar, too, found no phosphorylation of added pyruvate; phosphopyruvate appeared only when C₄-dicarboxylic acids were added as substrates. The new observation is that the specific

activity of phosphopyruvate is lower than that of inorganic phosphate, and also lower than that of the labile ATP phosphorus during the early stages of the incubation; further, that the turnover of phosphopyruvate is very high. The specific activity data indicate that phosphate does not react directly with the substrate. The low specific activity of phosphopyruvate at the early stages of incubation proves that the phosphate group is derived from a phosphate source other than inorganic phosphate. As ATP is the only alternative source present in sufficient quantities, it must be the phosphate donor. All data are in accordance with this conclusion.

The known pathway of formation of phosphopyruvate from glucose cannot account for the present observations. Lipmann (1941) and Meyerhof (1942) have considered an alternative pathway involving phosphorylation of a C_4 -dicarboxylic acid giving phosphoenoloxaloacetate, either directly or via phosphomalate and phosphofumarate. Phosphopyruvate was visualized as arising by decarboxylation from phosphoenoloxaloacetate. This assumption is perhaps at present the most useful working hypothesis, but the earlier schemes have to be modified by replacing ATP for phosphate as the phosphate donor. A direct phosphorylation of pyruvate is feasible under some conditions, as Lardy & Ziegler (1945) have demonstrated, showing the reversibility of the reaction

But the equilibrium constant of this reaction at pH 7.0 is about 7×10^{10} (see Meyerhof & Oesper, 1949; Burton & Krebs, 1953). Therefore no measurable quantities of phosphopyruvate could accumulate under the conditions of the present experiments. This is borne out by the fact that pyruvate, alone, without bicarbonate, does not yield phosphopyruvate.

The significance of the formation of phosphopyruvate remains to be established. It cannot be a step in oxidative phosphorylation, but it is plausible that the synthesis of phosphopyruvate, by a reaction other than the reversal of reaction (1), is a key step in the resynthesis of glucose from C_4 and C_3 compounds. Isotope data on the role of bicarbonate in the synthesis of glycogen (Solomon, Vennesland, Klemperer, Buchanan & Hastings, 1941) are in accordance with this concept.

SUMMARY

1. Phosphopyruvate is formed by washed, sheep-kidney particles during the oxidation of α -oxoglutarate, succinate, fumarate, malate, oxaloacetate, and pyruvate plus bicarbonate; it is not formed during the oxidation of pyruvate alone. This is in accordance with earlier observations of Kalckar (1939). Phosphate esters other than phosphopyruvate are formed during the later stages of the incubation.

2. After short periods the specific activity of phosphopyruvate is lower than that of phosphate. The data exclude phosphate, and suggest adenosine triphosphate as the immediate precursor of the phosphate group of phosphopyruvate.

3. The observations that phosphopyruvate rapidly reaches isotopic equilibrium with added $^{32}\text{PO}_4^{3-}$ and that added phosphopyruvate is rapidly removed indicates that the turnover rate of phosphopyruvate is high.

4. The mechanism of formation of phosphopyruvate is discussed. It is suggested that reactions of the type proposed by Meyerhof (1942) and by Lipmann (1941), involving C_4 -dicarboxylic acids, are probably implicated. The formation of phosphopyruvate may be a step in the synthesis of carbohydrate.

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