The Formation of Phosphopyruvate in Washed Particles of Kidney and Liver

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A formation of phosphopyruvate from C_4 dicarboxylic acids was first observed by Kalckar (1939). His findings were confirmed by Leloir & Munoz (1944) and Bartley (1954); adenosine triphosphate (ATP) was shown to be the phosphate donor (Bartley, 1954). In the present paper the conditions for the formation of phosphopyruvate have been studied in detail.

EXPERIMENTAL

Tissue preparations. These were usually twice-washed homogenates, i.e. mixtures of mitochondria and nuclei ('cyclophorase') prepared according to Bartley & Davies (1954). Sheep kidney was dispersed with a Waring Blender; rat and pigeon liver were homogenized in a stainless-steel homogenizer of the Potter-Elvehjem type. In a few experiments washed mitochondria were used; these formed about the same amount of phosphopyruvate as the cyclophorase preparations: the latter were used mainly because of the greater ease of preparation.

Media. In most experiments 2 ml. of the suspension (about 70 mg. dry wt.) were added to 2 ml. of a solution containing K phosphate buffer pH 7.4 (0.02M), Na adenosine triphosphate (0.002M), $MgCl_2$ (0.002M) and the oxidizable substrate, the amounts of which are given in the description of the individual experiments.

Special chemicals. ATP was prepared according to LePage (1949), phosphopyruvate according to Ohlmeyer (1951), 2-phosphoglyceric acid according to Kiessling (1935), phosphomalate according to Friedkin & Lehninger (1947), 3-phosphoglyceric acid according to Neuberg & Lustig (1942). Hydroxymaleic and hydroxyfumaric acids were made by the following modification of the method of Fenton & Jones (1900). A solution of DL-malic acid (26.8 g.) and ferrous sulphate (7 g.) in water (60 ml.) was frozen by standing the container in a mixture of solid CO_2 and ethanol. The mixture was vigorously stirred during freezing in order to prevent the formation of one solid block. Hydrogen peroxide (100 ml. of 20 vol.) was frozen in a similar manner and added in small portions to the mush of malic acid and $FeSO_4$ solution. If hydroxymaleic was required, the procedure was then as described by Fenton & Jones, i.e. ether extraction from 10% (w/v) H_2SO_4 solution of the crude product. To obtain hydroxyfumaric acid the

mixture was added to ice-cold H_2SO_4 (final concentration of H_2SO_4 , 30% w/v) in small portions. The mixture was allowed to warm to room temperature and then heated for 30 min. on a water bath at 40°. After cooling to room temperature the mixture was shaken with successive 50 ml. portions of ether (five or six extractions). The combined ether extracts were evaporated to dryness and the yellowish solid obtained was taken up in the minimum amount of warm acetone and reprecipitated by the addition of 3 times its volume of $CHCl_3$. The crude product (m.p. 168°) was dissolved in the minimum amount of boiling ethyl propionate and treated with charcoal. After filtration, while still hot, the substance was induced to crystallize by the gradual addition of $CHCl_3$ (about 3 vol.). Recrystallization from ethyl propionate and $CHCl_3$ gave hydroxyfumaric acid (m.p. 176° uncorr.). Both hydroxymaleic acid (m.p. 152°) and hydroxyfumaric acids were estimated by the aniline decarboxylation method (Krebs & Eggleston, 1945), according to which the hydroxyfumaric acid was 98.5% and the hydroxymaleic acid 96% pure. Another sample of hydroxymaleic acid was prepared and recrystallized (m.p. 151–152°) according to the method of Roberts (1952). Half of this material was dissolved in 30% (w/v) H_2SO_4 warmed to 40° for 30 min., cooled and extracted into ether. After evaporation of the ether the solid was dissolved in the minimum of acetone at 60° and treated with charcoal. The hydroxyfumaric acid was precipitated by the addition of 3 vol. of light petroleum (b.p. 60–80°) filtered off, dissolved in cold acetone and precipitated by light petroleum several times until the m.p. was 178°. The hydroxyfumaric acid was then twice recrystallized from ethyl acetate by the addition of light petroleum and dried.

Oxaloacetate in this paper refers to the equilibrium mixture of keto and enol forms produced when hydroxymaleic acid was dissolved in water, neutralized to pH 7.4, and allowed to stand for at least 5 min.

Other oxidizable substrates were commercial preparations; malic acid was added as the racemic mixture. Unless otherwise stated, substrates were neutralized to pH 7.4 with $N-NaOH$ before addition to the incubation medium. Other chemicals were of 'AnalaR' standard.

Analytical methods. The phosphorus-containing compounds were separated by paper chromatography, and determined as described by Bartley (1953).

The sum of (malate + fumarate) was estimated with washed suspensions of *Lactobacillus arabinosus* (Nossal, 1952) and keto acids by the 'specific extraction' procedure of Friedemann & Haugen (1943). Oxygen uptake was measured manometrically in the Warburg apparatus. A stick of yellow phosphorus was placed in the centre well in anaerobic experiments.

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Table 1. *Comparison of phosphopyruvate formation from different substrates*

Expt. 1. Rat-liver cyclophorase, about 60 mg. dry wt., incubated in Warburg vessels at 40° in O₂ for 15 min. Expt. 2. Pigeon-liver cyclophorase, about 60 mg. dry wt., incubated at 20° for 20 min. Each vessel contained 2 ml. of the tissue suspension in 0.9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7.4 (0.02M), Na adenosine triphosphate (0.002M), MgCl₂ (0.002M), 0.025M substrate and in Expt. 1 tracer amounts of ³²PO₄. The centre well contained 0.2 ml. of 2N-NaOH and filter paper. The phosphopyruvate formed in Expt. 1 was measured by determination of its radioactivity; in Expts. 2 and 3 the phosphopyruvate was measured, after isolating the substance on the paper chromatogram, by the phosphate content of the chromatogram spots.

Expt. no.	Tissue	Substrate	Oxygen consumption (μmoles)	Phosphopyruvate formed (μmoles)
1	Rat liver	Citrate	37.3	1.4
		α-Oxoglutarate	38.7	1.0
		Succinate	30.5	2.1
		Fumarate	18.9	3.2
		Malate	21.0	2.7
2	Pigeon liver	Malate	—	6.3
		Oxaloacetate	—	4.1
3	Pigeon liver	Malate	5.0	5.5
		Oxaloacetate	6.0	3.6

Measurements of radioactivity were made in a liquid counter (M6, 20th Century Electronics, Dunbar Street, West Norwood, London, S.E. 27) with the diluted paper digests. Counts were corrected for decay, background and dead time.

Calculation of amounts of phosphate esters from measurements of radioactivity. In the tissue suspensions used the ATP exchanged its labile phosphate groups with inorganic P very rapidly and within a few minutes specific activities became equal to that of the inorganic phosphate. Any organic P formed from ATP or directly from inorganic P thus had the same specific activity as P and the quantities of the organic P formed could be calculated by measuring the radioactivity of the substances after separation.

RESULTS

Comparison of phosphopyruvate formation from citrate, α-oxoglutarate, succinate, fumarate or malate. Earlier experiments had shown qualitatively (Bartley, 1954) that phosphopyruvate is formed when any tricarboxylic acid cycle intermediate or pyruvate and bicarbonate was incubated with respiring sheep-kidney cyclophorase preparations. The experiments recorded in Table 1 were designed to measure quantitatively the yield of phosphopyruvate in the presence of various precursors. Expt. 1 shows that during 15 min. of incubation the greatest quantity of phosphopyruvate was formed when fumarate or malate was the substrate; oxaloacetate was not tested in this experiment, but as shown by Expts. 2 and 3, and Table 4, it was rather less active in forming phosphopyruvate than malate or fumarate. This is discussed more fully later. Pigeon liver (about 60 mg. dry wt.) incubated at 20° for 20 min. accumulated about twice as much phosphopyruvate from malate (about 6 μmoles) as the same amount of rat liver incubated for 15 min. at 40° (about 3 μmoles). The amounts of phospho-

Table 2. *Comparison of phosphopyruvate and phosphoglycerate formation with varying concentrations of fumarate*

Rat-liver cyclophorase, about 50 mg. dry wt., incubated in Warburg vessels for 80 min. in O₂ at 20°. Each vessel contained 2 ml. of the tissue suspension in 0.9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7.4 (0.02M), Na adenosine triphosphate (0.002M), MgCl₂ (0.002M) and substrate. The centre well contained 0.2 ml. of 2N-NaOH and filter paper. The phosphate-containing compounds were measured, after isolation on the paper chromatogram, by determination of the phosphate content of the chromatogram spots.

Fumarate concentration (M)	O ₂ uptake (μmoles)	Phosphopyruvate formed (μmoles)	2- and 3-Phosphoglycerates formed (μmoles)
0.04	11.40	4.9	3.0
0.02	12.75	4.8	3.0
0.01	13.90	4.2	3.5

pyruvate (4–5 μmoles) formed from fumarate were not markedly altered by variation of the substrate concentration between 0.01 and 0.04M (Table 2). As described by Bartley (1954), other phosphate esters accumulate when incubation is prolonged. The main additional chromatogram spot (a mixture of 2 or 3 phosphoglyceric acids, see later) appears when the amount of phosphopyruvate becomes large. In the experiment described in Table 2 the final amounts of phosphoglyceric acids were 3–3.5 μmoles. The molar sum of phosphopyruvate and phosphoglycerates was about equivalent to half the molar amount of O₂ utilized (Table 2).

Anaerobic formation of phosphopyruvate from fumarate, malate or oxaloacetate. Under anaerobic conditions, with fumarate or malate as substrates, and fluoride (0.02M) as an inhibitor of adenosine-

triphosphatases, the maximum phosphopyruvate formation was less than 10 % of the aerobic formation, e.g. 70 mg. dry wt. of tissue particles formed 0.3 μ mole in 1 hr. Oxaloacetate yielded much more phosphopyruvate under these conditions (Table 3). The maximum amount of phosphopyruvate was found after 10 min. of incubation; if incubation was prolonged the substance disappeared. This was probably correlated with the decline of ATP concentration, which occurred in spite of the fluoride which was added to the incubation medium and the action of pyruvic phosphokinase. No phosphoglycerates were formed, presumably because of the inhibition of enolase by the fluoride.

Comparison of the aerobic formation of phosphopyruvate from malate or oxaloacetate. The relatively low rate of phosphopyruvate formation from oxaloacetate might be due to an inhibition by a high concentration of oxaloacetate. To test this, three flasks containing pigeon-liver cyclophorase and cofactors were set up; to one were added 25 μ moles of malate, to the second 25 μ moles of oxaloacetate and to the third oxaloacetate was added in portions of 0.5 μ mole at regular intervals over a period of 1 hr. to a total of 25 μ moles. The results given in Table 4 show that the rate of phosphopyruvate formation was higher at the higher oxaloacetate concentration. Thus oxaloacetate in the concentra-

Table 3. *Anaerobic formation of phosphopyruvate from oxaloacetate*

Sheep-kidney cyclophorase, about 60 mg. dry wt., incubated in Warburg vessels at 40° in N₂ with yellow phosphorus in the centre well. The 4 ml. of incubation mixture contained 2 ml. of the tissue suspension in 0.9 % (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7.4 (about 20 μ moles), Na adenosine triphosphate (about 5 μ moles), MgCl₂ (0.002 M), NaF (0.04 M) and oxaloacetate 0.025 M. The phosphate-containing compounds were measured after isolation on the paper chromatogram by determination of the phosphate content of the chromatogram spots.

Incubation time (min.)	ATP + ADP phosphate present (μ moles)	AMP phosphate present (μ moles)	Orthophosphate present (μ moles)	Phosphopyruvate formed (μ moles)
0	17.2	0	23.9	0
5	8.5	2.7	26.9	1.0
10	6.7	3.3	29.5	1.5
20	5.2	4.3	30.6	1.5
40	—	—	—	0

Table 4. *Comparison of phosphopyruvate formation from malate or oxaloacetate*

Pigeon-liver cyclophorase shaken in open flasks at 20°. Each flask contained 10 ml. of pigeon-liver cyclophorase in 0.9 % (w/v) KCl, and in Expts. 1 and 2, 10 ml. of a medium containing K phosphate buffer pH 7.4 (0.02 M), Na adenosine triphosphate (0.002 M), MgCl₂ (0.002 M) and 0.025 M substrate. In Expt. 3 the 10 ml. of cyclophorase was added to 9.02 ml. of a medium which contained initially only 2 % of the amount of substrate but which contained the same total quantity of the other cofactors. The remainder of the substrate was added in this experiment in portions of 0.02 ml. at 1 min. intervals throughout the incubation until the 500 μ moles of substrate had been added. In Expt. 1 the substrate was malate and in Expts. 2 and 3 it was oxaloacetate. 2.0 ml. samples of the incubation mixture were removed at the times stated below for analysis. The analytical data refer to the content in the 2 ml. sample.

Expt. no.	Incubation time (min.)	Malate present (μ moles)	Oxaloacetate present (μ moles)	Phosphopyruvate found (μ moles)	2- and 3-Phosphoglycerates found (μ moles)	Ratio: total μ moles substrate phosphorylated/ substrate not recovered as malate or keto acid $\times 100$
1	0	25	0	0	0	—
	15	18.8	0.52	2.15	1.1	57.2
	30	15.9	0.95	2.8	2.0	59.4
	45	14.0	0.64	—	—	—
	60	12.9	0.87	1.8	5.0	60.5
2	0	0	25	0	—	—
	15	3.52	18.0	1.2	1.1	63.3
	30	2.29	11.2	1.75	1.8	30.8
	45	1.32	7.1	—	—	—
	60	1.24	4.7	0.8	2.95	19.6
3	0	0	0.5	—	—	—
	15	0.22	4.47	0.25	0.3	—
	30	0.66	6.21	0.45	0.90	—
	45	2.49	10.3	—	—	—
	60	0.98	11.9	0.5	1.6	—

tion tested, did not inhibit phosphopyruvate formation. A remarkably high proportion (over 50 %) of the malate removed can be accounted for as phosphorylated products. In agreement with other experiments the formation of phosphopyruvate from oxaloacetate was lower than that with malate.

Phosphopyruvate formation in the presence of ketone-binding agents. Ketone-binding agents were added in order to test whether free oxaloacetate was an intermediate in the phosphopyruvate formation from malate. Table 5 shows the effect of semicarbazide on phosphopyruvate formation from malate by pigeon liver. In the first experiment phosphorylation was slightly reduced by the semicarbazide, but as the respiration was also inhibited in roughly the same proportion, this might be expected. In the second experiment the phosphorylation was slightly higher in the presence of the semicarbazide.

The experiments were repeated in the presence of other ketone-binding agents (hydroxylamine 0.005M or cyanide 0.01M); ferricyanide (0.01M) was added with the cyanide as hydrogen acceptor. Table 6 correlates oxygen and malate consumption and production of phosphopyruvate and of phosphoglycerate. Both semicarbazide and hydroxylamine approximately halved the oxygen uptake without greatly reducing either the malate consumption or the formation of phosphopyruvate and phosphoglycerate. With cyanide the malate consumption was approximately halved. Of special interest is the almost quantitative recovery of malate as phos-

phorylated products in the presence of potassium cyanide. Raising the cyanide concentration to 0.02M reduced the phosphorylation to about one-third. When oxaloacetate was the substrate no phosphopyruvate was formed in the presence of semicarbazide or of cyanide.

Phosphomalate as intermediate in the formation of phosphopyruvate. Lipmann (1941) suggested that phosphomalate is an intermediate in the formation of phosphopyruvate from C_4 acids. Phosphomalate was prepared by Friedkin & Lehninger (1947) and according to Friedkin (1951) phosphomalate is not oxidized by liver and does not inhibit the oxidation of malate. It was confirmed that no oxygen uptake occurs when phosphomalate is the sole substrate, but 0.0025M phosphomalate was found to reduce oxygen uptake and phosphorylation to about one-third when malate was the substrate (Table 7). To test the possibility of phosphomalate acting as an intermediate in phosphopyruvate formation, 2 μ moles of the substance were added to 4 ml. of a pigeon cyclophorase preparation oxidizing malate; isotopic phosphate was added to the incubation mixture which was shaken at 20° for 1 hr. The phosphorylated substances were separated on the paper chromatogram and their radioactivities were determined. The phosphopyruvate and phosphoglycerate formed were both in isotopic equilibrium with the inorganic phosphate, whereas the phosphomalate was inactive. Thus phosphomalate cannot be an intermediate in the formation of phosphopyruvate.

Table 5. *Phosphorylated products formed from malate with and without the addition of semicarbazide*

Pigeon-liver cyclophorase incubated in Warburg vessels in O_2 at 20°. Expt. 1: 0.0125M substrate, 84 mg. dry wt. of particles. Expt. 2: 0.0062M substrate, 0.02M THAM buffer pH 7.4, 96 mg. dry wt. of particles. Other additions as in Table 1.

Expt. no.	Addition	O_2 uptake (μ moles)	Phosphopyruvate formed (μ moles)	2- and 3-Phosphoglycerates formed (μ moles)
1	None	23.8	7.62	6.9
	Semicarbazide (0.04M)	18.5	3.98	6.05
2	None	—	5.46	1.63
	Semicarbazide (0.08M)	—	6.20	1.41

Table 6. *Phosphopyruvate formation from malate in the presence of ketone-binding agents*

Pigeon-liver cyclophorase incubated in Warburg vessels in O_2 at 20° for 1 hr. Malate initially present, 41.3 μ moles. The vessel with cyanide contained 0.01M ferricyanide as hydrogen acceptor; other additions as in Table 1.

Ketone-trapping agent added	O_2 consumption (μ moles)	Phosphopyruvate formed (μ moles)	2- and 3-Phosphoglycerates formed (μ moles)	Malate consumption (μ moles)	% of malate consumption recovered as phosphorylated compounds
None	21.8	7.78	5.40	17.5	75.5
Semicarbazide (0.08M)	11.1	8.85	3.42	17.7	69.5
Hydroxylamine (0.005M)	12.95	4.38	4.65	14.7	63.4
Cyanide (0.01M)	0	3.72	5.08	9.1	96.7

Comparison of reactivity of enol and keto oxaloacetate. The dehydrogenation of malate might produce one of the enol forms of oxaloacetate, hydroxyfumaric or hydroxymaleic acid, rather than the keto form. These three substances are mutually interconvertible:

but the rate of interconversion is unknown.

Some samples of hydroxymaleic and hydroxyfumaric acids showed differences in the rates at which they formed phosphopyruvate; these differences were reduced, though not abolished, when the hydroxyfumaric acid was recrystallized. This suggested that the sample of hydroxyfumaric acid might contain an inhibitor. Accordingly, another sample of hydroxymaleic acid was prepared and recrystallized (m.p. 151–152°) by the method of Roberts (1952). Half of this was converted into hydroxyfumaric acid as described in the Experimental section and these purified samples of hydroxymaleic and hydroxyfumaric acids were used in the following experiment. To avoid inaccuracies

due to the use of individual incubation vessels, samples were abstracted from a common incubation mixture. Four conical flasks of capacity 100 ml. were used. Each flask contained 49.5 mg. of the keto acid, one pair hydroxymaleic acid, one pair hydroxyfumaric acid. To one of each pair were added 15 ml. of a medium containing the usual cofactors and sufficient NaOH to neutralize the acid. The medium also contained aminotrihydroxymethylmethane (THAM) buffer (final conc. in medium 0.05M). The two flasks were shaken in a water bath at 20° for 6 min. to allow the keto–enol equilibrium to be established. Pigeon-liver cyclophorase suspension (15 ml.) was then added to each flask without stopping the shaking and a 4 ml. sample was immediately abstracted and pipetted into 0.5 ml. 30% (w/v) trichloroacetic acid TCA. Further samples were withdrawn after 2, 4, 6, 8 and 10 min. incubation. The other two flasks were treated in the same way, except that the cyclophorase was added immediately after the medium, without the 6 min. equilibration period. The TCA extracts were analysed for malate and phosphopyruvate. The results in Table 8 show that both hydroxymaleic

Table 7. *Comparison of oxygen uptake, malate consumption and phosphopyruvate formation with and without phosphomalate*

Pigeon-liver cyclophorase incubated at 20° in O₂ for 1 hr. Malate added 41.3 μmoles. Phosphomalate in Expt. 1, 0.0025M; in Expt. 2, 0.0005M. Dry weight of particles added, 84 and 75 mg. dry wt. respectively; other additions as in Table 1.

Expt. no.	Addition	O ₂ consumption (μmoles)	Malate consumption (μmoles)	Phosphopyruvate formed (μmoles)	2- and 3-Phosphoglycerates formed (μmoles)
1	None	24.8	—	7.6	6.9
	Phosphomalate (0.0025M)	7.9	—	3.2	1.8
2	None	21.8	17.5	7.78	5.4
	Phosphomalate (0.0005M)	20.9	15.2	9.58	4.08

Table 8. *Comparison of phosphopyruvate and malate production from hydroxymaleic acid, hydroxyfumaric acid and oxaloacetic acid formed from both these substances*

49.5 mg. of hydroxymaleic acid were weighed into each of a pair of 100 ml. conical flasks and the same amount of hydroxyfumaric acid into a further pair. To one of each pair of flasks were added 15 ml. of a medium containing K phosphate buffer pH 7.4 (0.02M), Na adenosine triphosphate (0.002M), MgCl₂ (0.002M), THAM buffer pH 7.4 (0.05M) and sufficient NaOH to neutralize the keto acid. The four flasks were shaken at 20° for 6 min. and medium was then added to the remaining two flasks. Pigeon-liver cyclophorase (15 ml.; 400 mg. dry wt.) was then immediately added to each flask. A 4 ml. sample of the mixture was immediately abstracted from each flask for analysis and further samples at the times stated below. The analytical values refer to content in the 4 ml. sample abstracted.

Time (min.)	Phosphopyruvate formed from				Malate formed from			
	Hydroxymaleic acid (μmoles)	Hydroxyfumaric acid (μmoles)	Oxaloacetic acid from hydroxymaleic acid (μmoles)	Oxaloacetic acid from hydroxyfumaric acid (μmoles)	Hydroxymaleic acid (μmoles)	Hydroxyfumaric acid (μmoles)	Oxaloacetic acid from hydroxymaleic acid (μmoles)	Oxaloacetic acid from hydroxyfumaric acid (μmoles)
2	0.74	0.58	1.00	0.92	0.44	0.24	0.64	0.46
4	1.20	1.32	1.68	1.76	1.26	1.00	1.36	1.26
6	1.60	1.52	2.10	2.20	1.72	1.28	1.82	1.88
8	2.08	1.86	2.66	2.86	2.16	2.04	2.50	2.46
10	2.32	2.50	2.92	2.90	2.68	2.82	3.08	2.96

and hydroxyfumaric acids formed phosphopyruvate at the same rate which was a little slower than the rate of phosphopyruvate formation from oxaloacetate. This would suggest that the keto form is the reactive tautomer. No clear distinction could be made in the rate at which malate was formed from the four substances.

Phosphorylated products formed from phosphopyruvate. The following evidence shows that the substance already referred to as 2- and 3-phosphoglycerates arises from phosphopyruvate.

A series of Warburg vessels were set up containing in the side arm about 3 μ moles of phosphopyruvate and 50 μ moles of fumarate and in the main compartment rat-liver cyclophorase, 1 μ mole of fumarate, the cofactors previously stated and isotopic orthophosphate. After 10 min. incubation in oxygen at 20° the contents of the side arm were tipped in, one of the vessels was removed and the reaction stopped by the addition of TCA. By this time all the exchangeable phosphate had equilibrated. The other vessels were incubated for a further 2, 4, 6, 8, 10, 15, 20, 30 or 60 min. The radioactivity incorporated into the phosphopyruvate and 2- and 3-phosphoglycerates at the various times was measured and the total quantities of each substance were estimated. Table 9 shows that in this experiment the disappearance of phosphopyruvate after 60 min. incubation was exactly balanced by the formation of 2- and 3-phosphoglycerates. Moreover, the specific activity of the 2- and 3-phosphoglycerates was lower at the end of the incubation

than at the time when inactive phosphopyruvate was added. This fall in specific activity could occur only if non-isotopic phosphate from phosphopyruvate was incorporated into 2- and 3-phosphoglycerates. Table 9 also shows that the formation of phosphopyruvate from fumarate was inhibited by the presence of preformed phosphopyruvate, presumably by mass action. New formation of phosphopyruvate did not occur until the initial concentration had fallen to about one-half. In a similar experiment summarized in Table 10 phosphopyruvate was added to a rat-liver suspension oxidizing citrate and measurements were made of the changes in the amount and specific activity of 2- and 3-phosphoglycerates with time. The amount of 2- and 3-phosphoglycerates increased with time, but the specific activity fell. As phosphopyruvate was the only source of non-isotopic phosphate, the decrease in specific activity of 2- and 3-phosphoglycerates means that it must have been formed from phosphopyruvate. Further evidence that 2- and 3-phosphoglycerates are formed from phosphopyruvate is presented in Table 11. It shows once again that when non-labelled phosphopyruvate is added to the incubation medium 2- and 3-phosphoglycerates contain phosphate of lower specific activity than the inorganic phosphate in the incubation medium. When 3-phosphoglyceric acid was added, the final product isolated in the 2- and 3-phosphoglycerates area of the chromatogram was radioactive, but the specific activity of the phosphopyruvate was less

Table 9. *Formation of 2- and 3-phosphoglycerates from phosphopyruvate by rat-liver cyclophorase*

Rat-liver cyclophorase incubated in Warburg vessels at 20° in O₂. The side arms of the vessels contained about 3 μ moles of phosphopyruvate and 50 μ moles of fumarate in 0.2 ml. water. The main compartments contained 2 ml. of cyclophorase (70 mg. dry wt.) in 0.9% (w/v) KCl, 1 μ mole of fumarate, 40 μ moles of phosphate containing tracer amounts of ³²PO₄, 4 μ moles of Na adenosine triphosphate and 4 μ moles of MgCl₂. Final volume 4 ml. The centre well contained 0.2 ml. of 2N-NaOH and filter paper. The vessels were incubated for 10 min. before the contents of the side arm were tipped; specific activities of orthophosphate, ATP and phosphopyruvate present after the 10 min. incubation but before tipping in the side arm contents are assumed to be the same, 3865 counts/min./ μ g. P. At the end of the incubation the specific activity of the phosphopyruvate was 1428 counts/min./ μ g. P and of 2- and 3-phosphoglycerates 931 counts/min./ μ g. P. The amount of radioactive material is calculated by dividing the total counts present in the isolated material at any time by the specific activity of the inorganic phosphate.

Time of incubation after tipping side arm contents (min.)	Phosphate of phosphopyruvate present		Phosphate of phosphopyruvate removed (μ g.)	Phosphate of 2- and 3-phosphoglycerates present	
	Radioactive (μ g.)	Non-radioactive (μ g.)		Radioactive (μ g.)	Non-radioactive (μ g.)
0	2.3	92.7	0	5.0	0
2	2.3	88.1	4.6	—	—
4	3.1	82.5	9.4	—	—
6	3.2	77.4	14.4	—	—
8	3.4	77.0	14.6	4.8	—
10	3.5	63.8	27.7	5.4	—
15	3.5	58.1	33.4	5.1	—
20	7.1	54.3	33.6	6.8	—
30	10.6	32.9	51.5	9.2	—
60	16.6	14.8	63.6	16.4	47.2

than that of the inorganic phosphate and must therefore contain some non-isotopic phosphate. This result indicates that the formation of 2- and 3-phosphoglycerates from phosphopyruvate is reversible.

Identification of 2- and 3-phosphoglycerates. In the solvent system used (*p*-toluenesulphonic acid-*tert.* amyl alcohol-water) the substance formed from phosphopyruvate and referred to as 2- and 3-phosphoglycerates runs in the same area as authentic specimens of 2-phosphoglyceric or 3-phosphoglyceric acids. No solvent system so far tested has

consistently separated the two phosphoglyceric acids. On a few occasions when a separation was achieved the substance formed from phosphopyruvate has been found in the area of 3-phosphoglyceric acid.

Formation of malate and fumarate from phosphopyruvate or 3-phosphoglyceric acid. The work of Utter & Kurahashi (1954) has demonstrated that the formation of phosphopyruvate from oxaloacetate is reversible. Table 12 demonstrates that phosphopyruvate or 3-phosphoglyceric acid can give rise to malate when incubated with pigeon-liver cyclophorase under anaerobic conditions. The amounts synthesized were small and were probably limited by the absence of suitable reducing systems in the washed homogenate. A small increase in the yield of malate was obtained by the addition of an artificial reducing system (dithionite) to the mixture. In this system very little malate is formed unless ADP is also added (Table 13).

Effect of fluoride on the interconversion of malate, phosphopyruvate and 3-phosphoglyceric acid. Phosphopyruvate was not formed from oxaloacetate under anaerobic conditions in the absence of fluoride. This is probably due to the fact that ATP required as phosphorylating agent is broken down by adenosinetriphosphatases in the absence of fluoride.

Fluoride (0.04M) reduced the oxygen uptake in the presence of malate by about 50% (Table 14). The formation of phosphopyruvate and of 2- and 3-phosphoglycerates is reduced to an even greater degree (Table 14, Expt. 4). Moreover, the proportion of 2- and 3-phosphoglycerates to phosphopyruvate is reduced by the presence of fluoride. This is to be expected if 2- and 3-phosphoglycerates are formed from phosphopyruvate by the action of enolase (Table 14).

Table 10. *Formation of 2- and 3-phosphoglycerates from phosphopyruvate by rat-liver mitochondria*

Rat-liver mitochondria incubated in Warburg vessels at 20° in O₂. The side arms of the vessels contained about 5 μmoles of phosphopyruvate in 0.5 ml. water. The main compartments contained 2 ml. of rat-liver mitochondria in 0.9% (w/v) KCl, 50 μmoles citrate, 40 μmoles of phosphate buffer, pH 7.4, containing tracer amounts of ³²PO₄, 4 μmoles of Na adenosine triphosphate and 4 μmoles of MgCl₂. Final volume 4 ml. The centre well contained 0.2 ml. of 2N-NaOH and filter paper. The vessels were incubated for 20 min. before the contents of the side arms were tipped. One vessel was immediately removed and the amount and specific activity of the 2- and 3-phosphoglycerates was measured. The incubation of the remaining vessels was terminated at the times given below.

Time of incubation after adding phosphopyruvate (min.)	Amount of 2- and 3- phosphoglycerates present (μmoles)	Specific activity of 2- and 3- phosphoglycerates (counts/min./μg. P)
0	0.77	230
5	0.85	215
10	0.95	203
25	1.42	122
35	2.20	76
55	3.24	52

Table 11. *Formation of 2- and 3-phosphoglycerates from phosphopyruvate in pigeon-liver cyclophorase*

Pigeon-liver cyclophorase (about 80 mg. dry wt.) incubated in Warburg vessels 1 hr. in O₂ at 20°. The 4 ml. of incubation medium contained 2 ml. of the tissue suspension in 0.9% (w/v) KCl, 2 ml. of a solution containing K phosphate buffer pH 7.4 (about 40 μmoles), Na adenosine triphosphate (about 4 μmoles), MgCl₂ (4 μmoles) and malate (42.3 μmoles) and the addition shown below. Tracer amounts of ³²PO₄ were added. The centre well contained 0.2 ml. 2N-NaOH and filter paper. The phosphate esters were separated on the paper chromatogram for determination of their phosphate content and of their radioactivity.

Expt. no.	Addition	Phosphopyruvate present			2- and 3-Phosphoglycerates present		
		Specific activity (counts/ min./μg. P)	Radio- active (μmoles)	Non-radio- active (μmoles)	Specific activity (counts/ min./μg. P)	Radio- active (μmoles)	Non-radio- active (μmoles)
1	Phosphopyruvate (4 μmoles)	1825	9.52	1.38	1610	3.71	1.14
	3-Phosphoglyceric acid (8 μmoles)	1990	9.70	0.50	960	4.34	5.16
2	Phosphopyruvate (5.75 μmoles)	390	4.06	0.76	392	7.88	1.47

Phosphopyruvate disappears on anaerobic incubation with pigeon-liver cyclophorase in the presence of ATP, and this disappearance is slightly inhibited by fluoride (Table 12). The removal is probably due to phosphopyruvic kinase and the fluoride effect can also be explained by an inhibition of ATPases. 3-Phosphoglyceric acid disappears on

incubation with pigeon-liver cyclophorase in the absence of fluoride. In the presence of fluoride, however, added 3-phosphoglyceric acid does not disappear (see Table 12). This is again in accordance with the assumption that the interconversion of phosphopyruvate and 3-phosphoglyceric acid in the cyclophorase preparations involves enolase.

Table 12. *Formation of malate by pigeon-liver cyclophorase from phosphopyruvate or 3-phosphoglyceric acid*

Pigeon-liver cyclophorase (about 70 mg. dry wt.) incubated in Warburg vessels at 20° in N₂ with phosphorus in the centre well. The 4 ml. of incubation mixture contained 2 ml. of the tissue suspension in 0.9% (w/v) KCl and 2 ml. of a solution containing K₂ phosphate buffer pH 7.4 (0.02M), Na adenosine triphosphate (0.002M), MgCl₂ (0.002M) and the substrate given below. Fluoride, when added, 0.0375M: Expts. 1 and 2 incubated 30 min.; Expt. 3, 60 min.

Expt. no.	Substrate added	Amount of substrate (μmoles)	Other additions	Malate formed (μmoles)	Phosphopyruvate present after incubation (μmoles)	2- and 3-Phosphoglycerates present after incubation (μmoles)
1	Phosphopyruvate	5.75	—	1.33	0	0.43
	Phosphopyruvate	5.75	Fluoride	1.21	0.55	0.33
	3-Phosphoglyceric acid	14.0	—	1.14	1.19	7.05
	3-Phosphoglyceric acid	14.0	Fluoride	0	0	14.0
2	3-Phosphoglyceric acid	28.0	—	1.33	—	—
	3-Phosphoglyceric acid	28.0	Fluoride	0.81	—	—
3	3-Phosphoglyceric acid	20.0	—	1.46	—	—
	3-Phosphoglyceric acid	20.0	Fluoride	0.71	—	—

Table 13. *Formation of malate from phosphopyruvate in the presence of dithionite*

Pigeon-liver cyclophorase (about 80 mg. dry wt.) incubated in Warburg vessels at 20° in N₂ with phosphorus in the centre well. The 4 ml. of incubation mixture contained 2 ml. of the tissue suspension in 0.9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7.4 (0.02M), MgCl₂ (0.002M), dithionite (30 μmoles) phosphopyruvate (6 μmoles) and adenosine diphosphate, when added, 15 μmoles.

Addition	Incubation time (min.)	Phosphopyruvate recovered (μmoles)	2- and 3-Phosphoglycerates formed (μmoles)	Malate formed (μmoles)
None	10	4.73	0.86	0
None	30	3.83	1.26	0.6
ADP	10	1.90	0.23	1.47
ADP	30	0.84	0.21	2.26

Table 14. *Effect of fluoride on oxygen consumption and phosphate esterification with malate as substrate*

Pigeon-liver cyclophorase incubated in Warburg vessels at 20° in O₂. The 4 ml. of incubation medium contained 2 ml. of the tissue suspension in 0.9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7.4 (0.2M), Na adenosine triphosphate (0.002M), MgCl₂ (0.002M) and 42.3 μmoles of malate. Fluoride, when added, 0.0375M final conc.; phosphopyruvate, when added, 10 μmoles. Expts. 1-3 contained about 70 mg. and Expt. 4 about 100 mg. dry wt. of cyclophorase.

Expt. no.	Addition	Incubation time (min.)	O ₂ uptake		Malate removed		Phosphopyruvate found		2- and 3-Phosphoglycerates formed	
			Without fluoride (μmoles)	With fluoride (μmoles)	Without fluoride (μmoles)	With fluoride (μmoles)	Without fluoride (μmoles)	With fluoride (μmoles)	Without fluoride (μmoles)	With fluoride (μmoles)
1	Phosphopyruvate	20	5.2	1.0	—	—	—	—	—	—
2	None	40	9.5	4.7	—	—	—	—	—	—
	Phosphopyruvate	40	7.2	4.0	—	—	—	—	—	—
3	None	90	16.5	10.9	—	—	—	—	—	—
	Phosphopyruvate	90	21.0	8.9	—	—	—	—	—	—
4	None	90	30.4	13.8	22.8	16.6	4.5	0.67	8.63	0.69
	Phosphopyruvate	90	30.8	15.1	23.2	15.1	4.82	1.45	9.35	0.84

DISCUSSION

Utter & Kurahashi (1954) have demonstrated the reactions

in isolated enzyme systems. Their findings satisfactorily account for the anaerobic formation of phosphopyruvate from oxaloacetate described in this paper. It remains to be explained why the aerobic rate of phosphopyruvate formation is greater from malate than from oxaloacetate. The lower reaction rate of oxaloacetate cannot be explained by an inhibitory effect of oxaloacetate since high concentrations of oxaloacetate do not inhibit phosphopyruvate formation. Moreover, it cannot be due to competition between oxidation and phosphorylation for the available oxaloacetate because, although a continuous regeneration of ATP is necessary for continued phosphopyruvate formation, the high concentration (0.0125M) of oxaloacetate present should be enough to saturate both enzyme systems.

The observations can be explained on the basis of two assumptions; first, that the substance phosphorylated is an oxaloacetate-enzyme complex and not free oxaloacetate; secondly, that the rate of formation of this complex from oxaloacetate is slow compared with the rate of its formation from malate. Moreover, if the attachment of oxaloacetate to the enzyme involved the ketone group, it would be understandable why ketone-binding agents have so little effect on phosphopyruvate formation from malate whilst completely inhibiting its formation from oxaloacetate.

The observations that phosphopyruvate and 2- or 3-phosphoglyceric acids are interconvertible in washed liver homogenates and that this interconversion is inhibited by fluoride imply the presence of enolase within the liver particles, and thus some of the reactions of glycolysis can occur here as well as in the extra-particulate sites of the cell. Enolase has been found in the nuclei of rat liver by Dounce (1950) and it is possible that the enolase activity in the pigeon-liver preparations is also due to the presence of nuclei.

SUMMARY

1. Respiring 'cyclophorase' preparations of sheep kidney, rat liver or pigeon liver form phosphopyruvate from citrate, α -oxoglutarate, succinate, fumarate or malate. The most active tissue was pigeon liver and the highest yields were obtained from fumarate or malate.

2. Under anaerobic conditions phosphopyruvate was formed from oxaloacetate, but not from malate or fumarate; under aerobic conditions malate and fumarate formed phosphopyruvate more rapidly than oxaloacetate.

3. The formation of phosphopyruvate from malate was not abolished by semicarbazide (0.08M), hydroxylamine (0.005M) or cyanide (0.01M). The major part of the malate used under these conditions was recovered as phosphopyruvate. Phosphopyruvate was not formed from oxaloacetate under these conditions. The interpretation of these facts is discussed.

4. Phosphomalate does not incorporate $^{32}\text{PO}_4$ in preparations forming phosphopyruvate and thus is not an intermediate in phosphopyruvate formation from malate.

5. Phosphate esters other than phosphopyruvate accumulate on prolonged incubation; these esters behave chromatographically like 2- and 3-phosphoglyceric acids. The formation of the esters is accounted for by the presence of a fluoride-sensitive enolase in the cyclophorase preparation.

6. Under anaerobic conditions the cyclophorase preparations form small quantities of malate from phosphopyruvate or 3-phosphoglyceric acid.

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