LXXVIII. THE NATURE OF PHOSPHORIC ESTERS FORMED IN KIDNEY EXTRACTS

By HERMAN KALCKAR

From the Institute of Medical Physiology, The University, Copenhagen

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SINCE the observation of the intense phosphorylation of glucose and glycerol in kidney extracts [Kalckar, 1937, 1] further investigations have been directed towards obtaining more information about the nature of the products of phosphorylation. The following organic substances are able to act as phosphate acceptors: adenylic acid, carbohydrates, glycerol and pyruvic acid.

EXPERIMENTAL

Methods. Inorganic P was estimated colorimetrically; acid-hydrolysable P was found by subtracting the inorganic P from the P present after 180 min. hydrolysis in *N* HCl at 100°. In the tables P determined is represented by P_0 , P_{60} , P_{90} or P_{180} , the subscript giving the number of min. of hydrolysis in boiling HCl. Triosephosphate (formed from hexosediphosphate in tissue extracts at 60°) was estimated as inorganic P after hydrolysis in *N* NaOH (P_{alk}). Phosphopyruvic acid was estimated as inorganic P after hydrolysis in *N*/30 alkaline iodine [Lohmann & Meyerhof, 1934]; triosephosphate is not hydrolysed in *N*/30 NaOH. The adenylypyrophosphate was estimated by means of its easily hydrolysable phosphate (7 min. in *N* HCl at 100°, designated by P_7). The fructose content was estimated by the method of Seliwanoff. The Seliwanoff reaction of fructosediphosphate is only 1/4 of that of free fructose. Dihydroxyacetonephosphate (or free dihydroxyacetone) was demonstrated by the method of Denigès [1909], i.e. instantaneous production of a purple substance in presence of conc. H_2SO_4 and morphine. Pyruvic acid was estimated by the method of Clift & Cook, using the modification of Elliott *et al.* [1935]: in presence of excess $NaHSO_3$ the pyruvic acid (and the trioses) react at room temperature to form bisulphite compounds.

The phosphorylated pyruvic acid was estimated by the $NaHSO_3$ method after liberation of the P group by boiling in *N* HCl for 60 min. Since the keto group is combined with P, phosphopyruvic acid should not bind $NaHSO_3$. The addition of Na_2HPO_4 in excess however will, in the presence of traces of I_2 , be able to liberate a definite amount of pyruvic acid which, converting I_2 into CHI_3 , liberates an equivalent amount of $NaHSO_3$. A sample of phosphopyruvic acid (Dr Lipmann) showed the following quantitative properties:

0.1 ml. solution contained 0.044 mg. iodine-labile P and exhibited by the $NaHSO_3$ method an I_2 consumption of:

0.20 ml. *N*/200 I_2 , directly.

0.62 ml. *N*/200 I_2 , after acid hydrolysis. Calc. 0.58 ml. *N*/200 I_2 .

Repeated estimations on this ester all show a directly determined I_2 consumption of the sample of about 0.20 ml. *N*/200 I_2 which is fairly independent of the absolute amounts of phosphopyruvic acid. (The preparation is free from triosephosphates). In Table XII I have therefore added 0.20 to the difference between P_{60} and P_0 as a correction.

Preparations. I am indebted to Dr P. Ostern for a large gift of pure adenylic acid and for some of the adenylypyrophosphate used in my experiments; another specimen of adenylypyrophosphate which I prepared by the method of Lohmann was 40 % pure. The fructose-6-phosphate (Neuberg ester) was made by the method of Neuberg [1918]. Phosphopyruvic acid was made by Dr Lipmann, to whom I express my thanks. The sample prepared by the synthetic method [Kiessling, 1936] was 40 % pure.

Experimental procedure. An aqueous extract of kidney cortex from rabbits or cats was used. The cortex was chopped in a cooled Latapie mill and the pulp was extracted for 5 min. with an equal volume of $M/30$ phosphate buffer (pH 8). After the extraction the mixture was centrifuged (cold) for 1–2 min.; the supernatant liquid which was red and turbid and without cell structure was used for the experiments.¹ 1 or 1.5 ml. fluid was pipetted into an ice-cooled manometer vessel which contained 0.2 ml. 2 % NaF. When the cortex was extracted with water the manometer vessel contained 0.5 ml. $M/15$ phosphate buffer (pH 8). The side bulb contained the phosphate acceptors (adenylic acid, sugars, glycerol etc.). In most of the experiments the vessel was filled with 100 % O_2 , the CO_2 formed being absorbed by KOH. The extracts used to demonstrate the formation of unknown phosphate acceptors from malate were dialysed. The apparatus used for dialysis was a tilting board to which a bag of cellophane was tied. However, since dialysing for 3–6 hr. even at 0° very frequently destroys the ability to esterify (addition of coenzymes did not activate such extracts) the dialysis was replaced in most experiments by a washing of the chopped tissue.

1. Adenylic acid as phosphate acceptor

The phosphorylation of adenylic acid through anaerobic and aerobic oxidoreductions has been observed in yeast juice, muscle extracts and erythrocytes. In kidney extracts the adenylic acid is phosphorylated only under aerobic conditions. The product of phosphorylation is probably adenylypyrophosphate. The results are summarized in Table I.

Table I. *Phosphorylation of adenylic acid*

Kidney from a fasted cat, extracted with $M/30$ Na_2HPO_4 . Per sample: 1.5 ml. extract, with the addition of 0.2 ml. 2 % NaF. Substrate: adenylic acid. Gas: 100 % O_2 .

	P_0	P_7	$P_7 - P_0$
(1) Temp. 37° . Incubation: 40 min.:			
Initial sample	1.28	—	—
Incub. without substrate	0.70	0.86	0.16
Incub. with adenylic acid (1.0 mg. organic P)	0.37	1.08	0.71
(2) Temp. 25° . Incubation: 60 min.:			
Initial sample	0.88	0.93	0.05
Incub. without substrate	0.60	0.89	0.29
Incub. with adenylic acid, 18 mg. (2.4 mg. organic P)	0.08	0.88	0.80

The samples incubated with adenylic acid exhibit an enormous increase in easily hydrolysable P, indicating formation of adenylypyrophosphate. As has been demonstrated for muscle extracts and yeast juice, adenylic acid acts as a system transferring P, the adenylypyrophosphate giving its labile P to carbohydrates. Similarly the transfer of P from adenylypyrophosphate to glucose might be a step in the phosphorylation of glucose in kidney extracts. It was, however, not possible to demonstrate a transfer of P from adenylypyrophosphate

¹ The R.Q. of such extracts is remarkably high, about 0.9 to 1 [Kalckar, *Enzymologia*, in the press].

to glucose in kidney extracts incubated under aerobic conditions (Table II), the P_7 value not decreasing in the presence of glucose.

Table II. *Addition of adenylypyrophosphate and glucose to minced kidney cortex*

Rabbit kidney, chopped. Per sample: 500 mg. tissue. Additions: 0.2 ml. 2% NaF, 0.5 ml. $M/15$ Na_2HPO_4 . Substrates: glucose, 40 mg. and adenylypyrophosphate ($P_7 - P_0 = 0.63$, $P_0 = 0.30$). Gas: 100% N_2 . Temp. 37°. Incubation: 60 min.

	P_0	P_7	$P_{\text{dephosphorylation}}$ (corr. for P_0 in "AP")
Initial sample	1.31	—	—
Incub. with adenylypyrophosphate	2.10	2.33	0.49
Incub. with adenylypyrophosphate + glucose	1.96	2.25	0.35

If $P_7 - P_0$ at $t=0$ was 0.63 mg. P the increases in P_0 agree fairly well with the decreases in the $P_7 - P_0$.

It is to be noticed that the adenylypyrophosphate is dephosphorylated in the incubated samples. Since the dephosphorylation of adenylypyrophosphate is not stopped by 0.2% NaF the rate of phosphorylation of adenylic acid must be considerably higher than the rate calculated directly from Table I.

2. Carbohydrates as phosphate acceptors

Glucose and fructose. The data already reported [Kalckar, 1937, 1] on phosphorylation in kidney tissue show that glucose is phosphorylated at a considerable rate. Table III shows that fructose is also phosphorylated.

Table III. *Phosphorylation of fructose*

Kidney from cat, extracted with water. Per sample: 1.5 ml. Additions of 0.2 ml. 2% NaF and 0.5 ml. $M/15$ Na_2HPO_4 . Substrate: fructose (30 mg.). Gas: 100% O_2 . Temp. 36°. Incubation: 30 min.

	P_0
Initial sample	1.30
Incub. without substrate	0.94
Incub. with fructose	0.72

The investigations have been extended to include the products of the phosphorylation. The formation of hexosediphosphate has been discussed [Kalckar, 1937, 1]. The formation of triosephosphate is demonstrable in ordinary trichloroacetic acid filtrates, if the kidney extract has been incubated for some minutes at 50–60° immediately before the precipitation of the proteins. At this temperature a considerable part of the hexosediphosphate is converted into alkali-labile triosephosphates, mainly dihydroxyacetonephosphate. From Table IV it

Table IV. *Formation of hexosediphosphate and dihydroxyacetonephosphate*

Kidneys from rabbit, chopped, the pulp washed with 2 vol. ice water, ground with sand, extracted with $M/15$ Na_2HPO_4 . Per sample: 1.5 ml. extract. Addition of NaF. Substrate: glucose (25 mg.). Gas: O_2 . Incub. 30 min. Temp. 37°.

	P_0	P_{alk}^*	Seliwanoff (mg. fructose)	Denigès (dihydroxy- acetone)
Initial sample	1.70	—	—	0
Incub. without substrate	1.50	1.57	—	0
Incub. with glucose	0.64	0.96	1.1	+++ (instantaneous)

* At the end of the experiment the incubated samples were heated to 60° for some minutes. Addition of ammonium molybdate to the trichloroacetic extract of the glucose sample did not produce any laevorotatory compound (phosphoglyceric acid).

appears that the ester formed in the sample incubated with glucose exhibits the properties of dihydroxyacetonephosphate, viz. an instantaneous intense Denigès colour reaction, and liberation of P by alkali.

In attempts at isolation of the ester formed in pulp of beef kidneys, a Ba salt with the properties of hexosediphosphate was found. The Ba salt was soluble in ice water, insoluble in hot water. Liberation of P after 3 hr. boiling in *N* HCl: 60%. Ratio H.J.-reduction/P=1.5/1,¹ i.e. hexose/P=3/1. Aldose: O. Seliwanoff: + + +. The liberation of P after 3 hr. hydrolysis is about 60%, the liberation of P from hexosediphosphate being about 80%. The other properties of the ester, however, agree completely with those of hexosediphosphate.

Phosphorylation of fructosemonophosphate. Fructosemonophosphate (Neuberg ester) added to kidney extracts acts as phosphate acceptor, yielding hexosediphosphate.

Table V. *Phosphorylation of fructose-6-phosphate*

Kidneys from cat, extracted with *M*/30 Na₂HPO₄. Per sample: 1.5 ml. extract. NaF added. Substrate: fructose-6-phosphate (Neuberg ester) equiv. 1.2 mg. organic P. Gas: O₂. Temp. 37°. Incubation: 17 min.

	O ₂ uptake μl.	P _o
Initial sample	—	1.10
Incub. without substrate	570	0.59
Incub. with Neuberg ester	435*	0.35
Incub. with glucose (30 mg.)	540	0.22
Incub. with glucose + Neuberg ester	440*	0.21

* The lower O₂ consumption in the samples incubated with Neuberg ester is due to a slight excess of oxalate after the precipitation of Ca.

As previously stated [Kalckar, 1938, 1], hexosediphosphate added to kidney extracts depresses the phosphorylation of glucose but not the phosphorylation of glycerol.

Galactose and arabinose. A slight phosphorylation takes place in the presence of galactose and arabinose (see Table VI). Phosphorylation of

Table VI. *Phosphorylation of galactose and arabinose*

Kidneys from cat, extracted with phosphate. Per sample: 1.5 ml. NaF added. Substrates: galactose (25 mg.) and arabinose (25 mg.). Gas: O₂. Temp. 36°.

	Min.	O ₂ uptake μl.	P _o
1. Initial sample	—	—	1.22
Incub. without substrate	30	460	1.12
Incub. with galactose	30	453	0.97
2. Initial sample	—	—	1.24
Incub. without substrate	40	535	1.03
Incub. with galactose	40	610	0.88
Incub. with glucose	40	545	0.60
3. Initial sample	—	—	1.20
Incub. without substrate	60	375	1.00
Incub. with arabinose	60	380	0.88

galactose in liver has been demonstrated by Kosterlitz [1937]. Phosphorylation of ribose has not been investigated, but it appears from the recent work of Dickens [1938] that ribosephosphate is more readily metabolized than arabinosephosphate. Starch is not phosphorylated in kidney extracts (see Table VII).

¹ 0.121 mg. H.J.-reduction corresponds to 0.082 mg. P.

Table VII. *Addition of starch to kidney extract*

Kidneys from a rabbit, extracted with phosphate. Per sample: 1.5 ml. NaF added. Temp. 37°. Substrates: glucose (in O₂). Starch (in N₂).

	Min.	O ₂ uptake μl.	P ₀
Initial sample	—	—	1.27
Incub. with glucose (aerobic conditions)	15	256	0.49
Incub. with starch (anaerobic conditions)	35	0	1.17

As reported [Kalckar, 1937, 1, 2], the phosphorylation of sugars is inhibited by small concentrations of phloridzin, an effect which is of importance in the discussion of the mechanism of active sugar absorption. The significance of the phosphorylation of carbohydrates will be discussed in another paper.

3. Glycerol as phosphate acceptor

If glycerol is added to kidney extracts under aerobic conditions an intense phosphorylation takes place (Table VIII).

Table VIII. *Phosphorylation of glycerol*

Kidneys (cat) minced, ground and extracted with phosphate. Per sample: 1.5 ml. extract. NaF added. Substrate: glycerol (20 mg.). Gas: O₂.

	Min.	O ₂ uptake μl.	P ₀	P ₁₈₀	P _{total}
Temp. 36°:					
1. Initial sample	—	—	1.22	—	—
Incub. without substrate	40	535	1.00	1.23	1.37
Incub. with glycerol	40	580	0.66	0.89	1.32
Incub. with glucose	40	530	0.60	1.14	1.37
			P ₀	P ₉₀	P _{total}
2. Initial sample	—	—	1.20	1.31	1.52
Incub. without substrate	60	275	1.00	—	—
Incub. with glycerol	60	330	0.61	0.83	1.59
Incub. under anaerobic conditions with glycerol	60	0	1.34	—	—
Temp. 22°:					
			P ₀	P ₆₀	
3. Initial sample	—	—	1.35	1.38	—
Incub. without substrate	60	—	0.89	—	—
Incub. with glycerol	60	—	0.52	0.78	—
Incub. with glucose	60	—	0.27	0.97	—

It will be noticed that the ester formed in the presence of glycerol is more acid-resistant than the ester formed in the presence of glucose. To determine whether the ester formed in the presence of glycerol is glycerophosphate or phosphoglyceric acid its isolation has been attempted. From 30 ml. of an extract from cat kidney incubated for 60 min. with glycerol (about 40–50 mg.) the Ba salt of an ester was prepared. Its precipitation required the addition of 3 vol. of alcohol to the neutralized trichloroacetic acid filtrate. The properties of the ester are as follows: Ba salt soluble in water, also in the presence of Ba(NO₃)₂, insoluble in alcohol. Quinine salt, needles, insoluble in cold, soluble in boiling, water; soluble in alcohol.¹ A solution of 22 mg. quinine salt in 2.5 ml. alcohol in a 2 dm. tube showed $\alpha_D = -2.78^\circ$, i.e. $[\alpha]_D = -158^\circ$. The ester is very resistant to acid hydrolysis—after 90 min. hydrolysis in boiling *N* HCl only 1–2% of the total P is liberated. Addition of ammonium molybdate did not produce any increased laevorotation. The properties described agree completely with laevorotatory α -glycerophosphate [Karrer, 1926].

¹ Quinine sulphate is insoluble in alcohol.

As described in another paper [Kalckar, 1939] the phosphorylation of glycerol is actually inhibited by α -glycerophosphate. According to Verzar & Laszt [1934] the phosphorylation of glycerol is of importance for the absorption of glycerol and fatty acids from the intestines.

4. Pyruvic acid as phosphate acceptor

Formation of phosphopyruvic acid from phosphoglyceric acid is a familiar intermediate reaction in muscle glycolysis. The formation of phosphopyruvic acid from inorganic P and pyruvic acid (formed from malic acid) is, however, a new observation.

Investigations of the action of malic acid on respiration and phosphorylation in kidney extracts led to the observation of formation of phosphopyruvic acid. If kidney extracts are dialysed for some hours and incubated with glucose a rather slight phosphorylation takes place; a sample incubated with malic acid, however, exhibits a very considerable phosphorylation (see Table IX).

Table IX. *Phosphorylations in presence of malic acid*

Rabbit kidneys, minced, extracted with 0.6% KCl solution. The extract is dialysed in cellophane. To the dialysed extract, phosphate (1.0 mg. P) and fluoride are added. Substrates: glucose and malate. Gas: O_2 . Temp. 36°.

	Min.	O_2 uptake μ l.	P_o
Dialysis, 3 hr. at 0°:			
Initial sample	—	—	1.10
Incub. with glucose + 0.4 mg. malate	125	338	0.89
Incub. with 10.0 mg. malate	110	885	0.38
Dialysis, 6 hr. at -1°:			
Initial sample	—	—	1.07
Incub. with glucose	90	115	1.11
Incub. with glucose + 10 mg. malate	90	388	0.77
Incub. with 10 mg. malate	90	387	0.81

Table IX shows that the phosphorylation in the presence of 10 mg. malic acid is very intense. Since the glycogen content of kidney extracts is very low and other carbohydrates are removed during dialysis, the intense phosphorylation in the presence of malic acid must be due to a phosphorylation of malate itself or more probably of an oxidation product of malate. The enormous increase of the respiration in the malate sample indicates an oxidation of malate to oxaloacetate. A further conversion of the oxaloacetate formed must, however, take place, since an accumulation of oxaloacetate will very soon inhibit the oxidation of malate. The O_2 consumption in the malate sample is, however, remarkably constant (Fig. 1).

It is probable that the oxaloacetate is decarboxylated to pyruvate which, when phosphorylated, yields phosphopyruvate. The experiments described and others similar have given many indications that malic acid oxidized in kidney extracts yields phosphopyruvic acid.

Investigations of the ester formed in the presence of malate proved that an ester is formed which has the properties of phosphopyruvic acid [Lohmann & Meyerhof, 1934] (see Table X).

Since the extracts are frequently inactivated during 3–6 hr. dialysis even at 0°, most of the experiments were made with extracts from washed tissue pulp. However, "washed" extracts contain more hexoses than dialysed extracts and a formation of hexosediphosphate is unavoidable.

Table X. *Formation of an iodine-labile phosphoric ester*

Rabbit kidney cortex was minced and washed in 3 vol. ice-cooled water; the washed tissue was then ground with sand and extracted with an equal vol. $M/30$ Na_2HPO_4 . 1.2 ml. extract were used. The mixture contained 0.2 % fluoride. Substrates: fumarate (30 mg.), malate (20 mg.) and glucose (30 mg.). Gas: O_2 . Temp. 37° . Incubation: 30 min.

	P_0	P_{alk}	P_{iod}	P_{merc}	P_{30}	O_2 uptake $\mu\text{l.}$
Initial sample	1.08	1.14	1.11	1.10	—	—
Incub. without substrate	0.96	1.05	1.00	1.00	1.14	230
Incub. with fumarate	0.64	0.70	0.96	0.96	1.13	432
Incub. with glucose	0.58	0.71	0.60	0.60	—	165
Initial sample (+ malate)	1.16	1.17	1.18	—	1.16	—
Incub. without substrate	1.00	1.00	1.02	—	1.11	474
Incub. with malate	0.78	0.78	0.98	1.00	1.12	720
Incub. with glucose (40 mg.)	0.75	0.83	0.77	0.75	0.96	323

P_0 : P determined directly.

P_{alk} : P determined after 20 min. incubation in $N/1$ NaOH .

P_{iod} : P after 20 min. incubation in $N/30$ alkaline iodine.

P_{merc} : P after 10 min. incubation in neutral HgCl_2 .

P_{30} : P after 30 min. hydrolysis in $N/1$ HCl at 100° .

Fig. 1. Rate of O_2 consumption of the incubated samples in Table X. — with 0.4 mg. malate. - - - - - with 10.0 mg. malate.

An examination of the pyruvic acid content showed that the malate samples always contain small amounts of pyruvic acid¹ and after 60 min. hydrolysis in boiling N HCl considerable amounts of pyruvic acid are liberated.

The difference between the contents of pyruvic acid in the samples hydrolysed with acid and the samples determined directly (and corrected, cf. Methods) gives the amount of phosphorylated pyruvic acid, a value which agrees well with that calculated from the amount of P liberated in $N/30$ alkaline iodine (cf. Table XI).

Attempts were made to isolate the ester formed in presence of malate. In extracts of beef or cat kidneys these were never successful, probably because the preparation of extracts from such large amounts of tissue takes so long that the enzymes are inactivated. An isolation from 4 exp. with rabbit kidneys (cf. Table XI) was therefore undertaken.

The extracts from 4 exp. were combined, the total content of I_2 -labile ester being about 40 mg. as Ba salt. The separation of the Ba salt of the I_2 -labile ester from the Ba salts of those phosphoric esters formed in the extracts without

¹ No accumulation of oxaloacetic acid was detectable by the method of Ostern.

Table XI. *Liberation of pyruvic acid from the iodine-labile phosphoric ester*

I. Kidneys from a fasted rabbit are chopped, washed and extracted with phosphate. Per sample (ml. extract): initial sample 1 ml., sample incubated without substrate. 1 ml., sample incubated with malate 10 ml.* NaF, 0.2%. Substrate: malate (150 mg.). Gas: O₂. Incubation: 45 min. Temp. 38°.

	P ₀ /ml.	P _{iod} /ml.	P _{iod} - P ₀ /ml.	NaHSO ₃ (ml. N/200 I ₂)	
				Direct	45 min. hydrolysis
Initial sample (15 mg. malate)	0.286	0.285	0	0.04	—
Inc. without substrate	0.230	0.230	0	0.04	—
Inc. with malate (150 mg.)	0.166	0.229	0.063	0.44	1.11

0.063 mg. I₂-labile P corresponds with 0.178 mg. pyruvic acid. The NaHSO₃ combined with 0.178 mg. pyruvic acid consumes: 0.80 ml. N/200 I₂. Found in the above exp. (1.11 - 0.44) + 0.20 (correction) = 0.87 ml. N/200 I₂.

* For isolation experiments.

II. Kidney extract (rabbit) prepared and incubated in the same manner as in exp. I. The sample with malate is incubated in 15 ml. extract. Substrates: lactate (20 mg.) and malate (20 mg.). Temp. 37°. Incubation: 45 min.

Initial sample	0.333	0.320	-0.013	0.08	0.33
Incub. without substrate	0.260	0.263	0.003	—	0.28
Incub. with lactate	0.260	0.258	-0.002	0.18	0.50
Incub. with malate	0.214	0.264	0.050	0.40	1.13

0.050 mg. P corresponds with 0.145 mg. pyruvic acid, equiv. 0.64 ml. N/200 I₂. Found in exp.: (1.13 - 0.40) + 0.20 (corr.) - 0.25 (initial diff.) = 0.68 ml. N/200 I₂.

III. Kidney extract (rabbit) prepared and incubated as in exps. I and II. The sample incubated with malate contains 10 ml. extract. Substrate: malate, 20 mg. Temp. 37°. Incubation: 50 min.

Initial sample	0.307	0.306	—	—	—
Incub. without substrate	0.214	0.215	—	—	—
Incub. with malate	0.142	0.214	0.072	0.32	0.92

0.072 I₂-labile P corresponds to 0.205 mg. pyruvic acid. The NaHSO₃ compound consumes 0.90 ml. N/200 I₂. Found: (0.92 - 0.32) + 0.20 = 0.82 ml. N/200 I₂.

malate (probably hexosediphosphate) was, however, impossible, since the solubilities of the Ba salts were the same (insoluble in hot water, sparingly soluble in cold water, soluble in dilute acids). According to Kiessling [1936] the Ba salt of phosphopyruvic acid, like the Ba salt of hexosediphosphate, is sparingly soluble in cold water and insoluble in hot water.

The sparingly soluble Ba salt showed a satisfactory agreement between the amount of pyruvic acid determined from the NaHSO₃ method and that calculated from the liberation of P in N/30 alkaline I₂.

From Table XII it appears that the ratio, pyruvic acid after acid hydrolysis to phosphate hydrolysed in alkaline iodine is about 3 : 1.

Table XII

For 0.5 ml. of a solution containing 15 mg. Ba salts the following values were found:

	mg. P	P _{iod} - P _{alk}	ml. N/200 I ₂ (equiv. NaHSO ₃ compound)
Directly	0.018	—	0.40
Hydrolysis in N/1 NaOH	0.020	—	—
Hydrolysis in N/30 alkaline I ₂	0.230	0.210	—
Hydrolysis in N/1 HCl for 60 min.	0.272	—	3.00
Control	—	—	0.02

0.21 mg. I₂-labile P corresponds to 0.61 mg. pyruvic acid. 0.61 mg. pyruvic acid (NaHSO₃) consumes 2.78 ml. N/200 I₂. Experiment shows: 2.98 ml. N/200 I₂.

Table XIII. *Identity with phosphopyruvic acid of the ester formed in presence of malate*

	Solubility of Ba salt	Alkali	Liberation of P in			Pyruv./P 3:1
			Iodine	HgCl ₂	N HCl	
Unknown P ester	Soluble in dilute acids, insoluble in hot water	No	Complete	Complete	Complete	3:1
Phosphopyruvic	Ditto.	No	Complete	Complete	Complete	3:1

In Table XIII the properties of the new ester are summarized and compared with the properties of phosphopyruvic acid.

Since all properties of the new ester agree with those of phosphopyruvic acid we conclude that malic acid in kidney extracts is oxidized to oxaloacetic acid which is then decarboxylated and phosphorylated. That the phosphopyruvic acid was formed from hexosediphosphate is impossible since the intermediate product, phosphoglyceric acid, cannot be converted into phosphopyruvic acid in presence of *N*/20 fluoride. In addition no formation of phosphoglyceric acid takes place in extracts incubated with glucose.

I was never successful in bringing about a formation of phosphopyruvic acid from lactic acid,¹ pyruvic acid or alanine, although this might perhaps be possible in kidney and liver slices.

Without doubt the formation of phosphopyruvic acid from malic acid is related to the synthesis of sugars from the fumaric acid system. A considerable increase in the glycogen content of the livers of animals fed with fumaric or succinic acid is well established [Stöhr, 1933; Blixenkrone-Møller, 1938]. In experiments with kidney slices Benoy *et al.* [1937] have demonstrated a definite formation of sugar from lactic and malic acids.

The pathway of sugar formation from lactic acid has been cleared up to a considerable extent by the studies of the Cambridge school [Green *et al.* 1937]; the understanding of the mechanism of this reaction we owe to the recent researches of the Heidelberg school [Meyerhof *et al.* 1938, 2]. Sugar formation from lactic acid is in all its properties a reversed glycolysis. Not only is the oxido-reduction the reverse of that in the glycolysis, but, as demonstrated recently by Meyerhof and co-workers, the transfer of P takes place in an exactly inverted manner. As we know, the adenylic acid in the glycolysis is rephosphorylated in two different ways: (1) through a direct P transfer from the phosphopyruvic acid (Parnas reaction); (2) through an esterification of inorganic P, a synthesis which is coupled to the reduction of cozymase by triosephosphate [Meyerhof *et al.* 1938, 1]. Very recently Lipmann [1939] has demonstrated a coupling between the reduction of aneurin pyrophosphate by pyruvic acid and the phosphorylation of adenylic acid. In the formation of sugar from lactic acid we have, in quite an analogous manner, a dephosphorylation of adenylypyrophosphate by two different reactions: (1) the direct P transfer from adenylypyrophosphate to pyruvic acid; (2) the liberation of inorganic P from adenylypyrophosphate, a reaction which is coupled to the oxidation of hydrocozymase by phosphoglyceric acid [Meyerhof *et al.* 1938, 2].

Sugar formation in animal tissues is an internal oxido-reduction which uses on a large scale the energy stored in the adenylypyrophosphate; respiration is therefore necessary for rephosphorylation of the adenylic acid. Such an

¹ According to Green [1936] and to Das [1937] the oxidation of lactic acid is more sensitive than oxidation of malic acid to tissue mincing.

aerobic rephosphorylation of adenylic acid has actually been demonstrated in kidney extracts.

What is the position of phosphopyruvic acid in the synthesis of sugar from lactic acid? According to Green and to Meyerhof there are three essential reactions:

(1) Lactic acid + phosphoglyceric acid + adenosinetriphosphate $\rightleftharpoons$ pyruvic acid + phosphotriose + adenosinediphosphate + P.

(2) Pyruvic acid + adenosinediphosphate $\rightleftharpoons$ phosphopyruvic acid + adenylic acid.

(3) Phosphopyruvic acid $\rightleftharpoons$ phosphoglyceric acid.

Whereas reactions (1) and (3) have been demonstrated experimentally in both directions, reaction (2) has so far not been demonstrated in the direction from left to right. Probably the accumulation of phosphopyruvic acid by addition of fumaric or malic acid to kidney extracts poisoned with fluoride corresponds to reaction (2) in the direction from left to right, occurring in conjunction with an aerobic rephosphorylation of the adenylic acid. According to recent investigations [Meyerhof *et al.* 1938, 3] in which labelled P was used, it seems that the reaction (2) actually proceeds only in the direction from right to left. The enzyme system of the phosphorylation of pyruvic acid in kidney extracts therefore cannot be identical with the enzyme system of the reaction: phosphopyruvic acid + adenylic acid $\rightleftharpoons$ pyruvic acid + adenosinediphosphate. Perhaps the oxidation of fumaric acid yields not $\text{CH}_3\text{CO}\cdot\text{COOH}$ but another acid which is the P acceptor proper.

SUMMARY

1. The products formed by esterification of various P acceptors are described. Adenylic acid is phosphorylated to adenylypyrophosphate. Glucose, fructose and fructose-6-phosphate are phosphorylated to fructosediphosphate and dihydroxy-acetonephosphate. Glycerol is phosphorylated to glycerophosphate. Since the quinine salt of the phosphoric ester showed a high laevorotation, $[\alpha]_D = -158$, the glycerophosphate accumulated is the laevorotatory α -glycerophosphate.

2. In extracts from washed tissue pulp or in dialysed extracts an intense phosphorylation very frequently takes place if fumaric or malic acid is added to the extract. These dicarboxylic acids increase the phosphorylation not only by stimulation of the respiration, but more probably by forming a phosphate acceptor. Probably a product formed by oxidation of malic or fumaric acid, must be assumed to act as phosphate acceptor. The phosphoric ester formed in the presence of malic or fumaric acid exhibits the typical properties of phosphopyruvic acid: liberation of P in *N*/30 alkaline I_2 and in neutral HgCl_2 , liberation of pyruvic acid after 60 min. hydrolysis in *N* boiling HCl . The Ba salt of the I_2 -labile phosphoric ester has been isolated in an impure state. The ratio of pyruvic acid after acid hydrolysis to P hydrolysed in alkaline I_2 was about 3 : 1.

The relation of the formation of phosphopyruvic acid from malic acid to sugar formation from malic acid has been discussed.

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