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Metabolism of Thiamine Phosphates in Washed Suspensions of Kidney Particles

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Whilst it is certain that the oxidation of pyruvate and α-oxoglutarate in animal tissues requires, among other cofactors, cocarboxylase (Lohmann & Schuster, 1937; Barron, Goldinger, Lipton & Lyman, 1941; Stumpf, Zarudnaya & Green, 1947), the precise role of this coenzyme is still obscure. As the oxidative decarboxylation of α-ketonic acids is accompanied by the synthesis of pyrophosphate bonds of ATP, it seemed possible that cocarboxylase might act as a phosphate carrier (Kiessling & Lindhal, 1952). This hypothesis became capable of experimental testing with the availability of isotopic phosphorus and of methods capable of separating small quantities of organic phosphates by paper chromatography (Hanes & Isherwood, 1949; Eggleston & Hems, 1952; Bartley, 1953*b*; Krebs & Hems, 1953). Experiments are reported in this paper in which the rate of incorporation of $^{32}PO_4$ into thiamine phosphates was measured and compared with the rate of oxidation of α-ketonic acids.

EXPERIMENTAL

Special chemicals. Thiamine triphosphate, thiamine diphosphate, oxythiamine diphosphate and thiamine were commercial samples (Roche Products Ltd.) of more than 95% purity. Thiamine monophosphate was prepared by the method of Karrer & Viscontini (1946) and oxythiamine by the method of Rydon (1951). Adenosine triphosphate (ATP) preparations contained about 80% ATP and 10% adenosine diphosphate (ADP). α-Oxoglutarate was prepared by the method of Friedman & Kosower (1946) and was not less than 98% pure, as estimated by the method of Krebs (1950). Pure sodium pyruvate was prepared according to Robertson (1942). All other reagents were of analytical grade.

Chemical estimations. Keto-acids were estimated by the 'specific' extraction method of Friedemann & Haugen (1943), succinate manometrically according to Krebs (1937), and free thiamine directly in the trichloroacetic acid (TCA) fitrates by the method of Melnick & Field (1938), the optical density of the red colour extracted into xylene being measured spectrophotometrically at 520 mμ. Measurements of radioactivity were made on a sample of the diluted, wet-ashed material or on the *iso*butanol extract obtained by the phosphate estimation. An M/6 liquid counter (20th Century Electronics) was used.

Preparation and incubation of the particulate suspensions. Particulate suspensions of sheep-kidney cortex, consisting mainly of mitochondria suspended in 0·9% (w/v) KCl, were prepared as described by Bartley (1953*a*). In all cases the 'R_3 residue' was used. Samples (2 ml.) of the tissue suspension (60–100 mg. dry wt.) were added to 2 ml. of medium in the main compartment of conical Warburg vessels which included ATP, $MgCl_2$ (both at 0·001 M final concn.) and phosphate buffer, pH 7·4 (final concn. usually 0·005 M). The centre wells contained NaOH and filter paper, with O_2 in the gas space. The O_2 uptake was measured at 40° and the incubation was stopped at varying times by transfer of the vessel to ice-water and addition of 0·5 ml. 30% (w/v) TCA to each vessel. The precipitated protein was removed by centrifugation and the TCA extract was stored at −18° until it could be analysed.

Paper-chromatographic separation, identification and estimation of thiamine phosphates. The method of Viscontini, Bonetti, Ebnöther & Karrer (1951) proved unsuitable, as it failed to separate thiamine mono- and di-phosphates, and a new method was therefore elaborated. Whatman no. 1 filter paper washed with ethylenediaminetetraacetic acid (Eggleston & Hems, 1952) was used throughout. Of several solvent systems tested, namely benzyl alcohol/formic acid, cellosolve/water, dioxan, *tert.*-pentyl alcohol/formic acid, *iso*propyl ether/formic acid and *p*-toluenesulphonic acid/*tert.*-pentanol/water (Hanes & Isherwood, 1949), only the last was satisfactory. Solutions were delivered from an Agla syringe (Burroughs Wellcome Ltd.) either as spots or as bands along a line 8 cm. from the end of a rectangular filter paper, 18 × 45 cm. After drying in a current of cold air, the papers were irrigated with the solvent by descending

chromatography in an all-glass apparatus, usually for 17 hr. Three methods were used to render thiamine derivatives visible on the paper. (1) Examination in ultraviolet light (260 mμ.). This is unspecific and, moreover, the absorption of small amounts of thiamine derivatives was obscured by the absorption of the *p*-toluenesulphonic acid in the solvent system. (2) Spraying with the molybdate reagent of Hanes & Isherwood (1949) which is applicable to phosphorylated derivatives. (3) Using the colour reaction with potassium bismuth iodide (Naimans, 1937) which is relatively specific for thiamine compounds. The spray reagent, which keeps indefinitely, is prepared by boiling about 5 g. of potassium bismuth iodide (B.D.H. reagent grade) in 100 ml. distilled water containing 0·5 ml. conc. HCl and filtering the orange solution when cold. When the dried chromatograms are sprayed with the reagent, thiamine derivatives appear as red-purple to orange spots, the tint varying with the compound. The chromatograms are viewed against a white light while still wet, as the red colour of the thiamine spots tends to be obscured by the orange background after drying. To obtain maximum colour development it is an advantage to re-spray the paper after drying. The colour of the background of the dried chromatogram may be removed by laying the paper in a photographic developing dish containing diethyl ether; a little water is added after immersion of the paper and the dish is gently rocked. The potassium bismuth iodide is, under these conditions, extracted by the ether layer and leaves behind the red or orange thiamine

Table 1. *Values of R_F, $R_{thiamine}$, and colour reactions with potassium bismuth iodide of some thiamine derivatives and of some other nitrogen-containing substances*

(5 μl. of approximately 0·05 M solutions of the substances were chromatographed as described in the text. The colours recorded were those observed by viewing in a white light. Substances were commercial preparations and in some cases contained other substances; the major constituent is indicated by an asterisk and the components are labelled in sequence *a*, *b*, etc. As the R_F values are somewhat variable, the $R_{thiamine}$ (distance travelled by substance/distance travelled by thiamine) values are also given.)

Substance	R_F	$R_{thiamine}$	Appearance under u.v. light	Colour when sprayed	Colour after washing, as described in text
Thiamine triphosphate	0·08	0·11	Absorbs	Orange	Orange
Thiamine diphosphate (cocarboxylase)	0·18	0·25	Absorbs	Orange-pink	Orange-pink
Oxythiamine diphosphate	0·11	0·16	Absorbs	Orange	Orange
Thiamine monophosphate	0·42	0·59	Absorbs	Pink-orange	Pink-orange
Thiamine	0·71	1·00	Absorbs	Pink	Pink
Oxythiamine	0·41	0·58	Absorbs	Pink-purple	Pink-purple
Chlorothiamine	0·82	1·15	Absorbs	Purple-pink	Purple-pink
5-(β-Hydroxyethyl)-4-methylthiazole	0·64	0·90	Absorbs	Faint purple-pink	None
5-Ethoxy-4-methylthiazole	0·64	0·90	Absorbs	Transient pink	None
2:4-Dimethyl-5-(β-hydroxyethyl)-thiazole. Two components *a**	0·65	0·91	Absorbs	Pale-pink	Almost invisible, pale-pink
2:4-Dimethyl-5-(β-hydroxyethyl)-thiazole. Two components *b*	0·87	1·22	Absorbs	Pale-pink	Almost invisible, pale-pink
5-(β-Acetoxyethyl)-4-methylthiazole	0·72	1·01	Absorbs	Very faint pink	None
4-Amino-5-cyano-2-methylpyrimidine	0·45	0·63	Faintly absorbs	None	None
Pyrimidine	0·53	0·75	Absorbs	None	None
4-Amino-2-methyl-5-thioformylamino-methylpyrimidine	0·75	1·05	Absorbs	Pink	Pink
Caffeine	0·77	1·08	Absorbs	None	None
Morphine sulphate. Two components *a**	0·68	0·96	Absorbs	Red	None
Morphine sulphate. Two components *b*	0·85	1·20	Absorbs	Red	None
Quinine hydrochloride	0·80	1·12	Intense-white fluorescence	Pink-orange	Pink-orange
Strychnine hydrochloride	0·82	1·15	Absorbs	Orange	Orange
Brucine hydrochloride	0·68	0·95	Absorbs	Orange	Orange
Cinchocaine hydrochloride	0·85	1·20	Intense-white fluorescence	Red	Red
Mepyramine	0·78	1·09	Intense-white fluorescence	Red	Red
Quinoline hydrochloride. Two components *a**	0·68	0·96	Intense-white fluorescence	Pink	Pink
Quinoline hydrochloride. Two components *b*	0·84	1·18	Intense-white fluorescence	Pink	Pink
Nicotine	0·55	0·77	Absorbs	Red-purple	Faint red
Choline chloride	0·45	0·63	Not visible	Purple	Purple-brown
Acetyl choline chloride	0·53	0·75	Not visible	Purple-blue	None
Benzoyl choline chloride. Three components *a*	0·4	0·57	Not visible	Purple	Purple-brown
Benzoyl choline chloride. Three components *b**	0·76	1·07	Fluorescence	Pink	Orange
Benzoyl choline chloride. Three components *c*	0·94	1·32	Not visible	Pink	Pink
Betaine hydrochloride	0·38	0·75	Slightly absorbs	Pale-blue	None
Agmatine sulphate	0·18	0·24	Very faintly absorbs	None	None

bismuth iodide complex. No extraction into the ether occurs until the water is added. The papers may be dried and kept as records. The method is not suitable if analysis of the phosphate on the chromatogram is required as some phosphate compounds disappear during the washing procedure. As little as 0·1 μg. of thiamine can be detected by this method. The substances tested with the potassium bismuth iodide reagent are listed in Table 1. Several compounds containing basic nitrogen react and the colour obtained appears to vary with the basicity of the nitrogen, becoming bluer the more basic the nitrogen, the bluest being obtained with the quaternary ammonium derivatives, such as choline. Esterification of choline with benzoic acid (benzoyl choline), which reduces the basicity of the nitrogen, changes the colour to red. In the experiments described in this paper, thiamine compounds were the only substances reacting with potassium bismuth iodide.

Of the three methods for locating the thiamine derivatives, the ultraviolet absorption has the advantage that the material remains unchanged and may be eluted from the paper for chemical estimations. The papers sprayed with molybdate may be used for phosphorus determinations as described by Bartley (1953*b*) and the papers treated with the potassium bismuth iodide reagent may subsequently be sprayed with molybdate and used for the determination of phosphorus, as potassium bismuth iodide does not interfere. The phosphate-containing compounds separated on paper were analysed as described by Bartley (1953*b*).

RESULTS

Effect of thiamine derivatives on respiration. The addition of thiamine, thiamine monophosphate, cocarboxylase, oxythiamine, oxythiamine diphosphate (all 0·001 M) or of thiamine, thiamine monophosphate or cocarboxylase (0·005 M) did not markedly alter the respiration or the ratio of oxygen uptake/pyruvate consumed ('O_2/pyruvate ratio') of a sheep-kidney suspension oxidizing pyruvate, but the addition of 0·005 M thiamine triphosphate inhibited respiration by 40% without altering the above ratio.

Metabolism of cocarboxylase during the oxidation of pyruvate. When cocarboxylase was incubated with sheep-kidney suspensions, cocarboxylase disappeared and other phosphate-containing compounds were formed. A quantitative experiment is shown in Table 2. Three new phosphorus-containing compounds were present at the end of the incubation in addition to the ATP, cocarboxylase and inorganic orthophosphate added to the incubation mixture. The six phosphorus compounds present are numbered in Table 2 on the basis of the R_F values. In another experiment in which the sodium bicarbonate concentration was lowered to 0·01 M, spots 1 and 4 were not formed and the amount of cocarboxylase broken down was much greater in the absence of pyruvate than in its presence (see Table 3).

Spot 3 behaved like a pure sample of thiamine monophosphate in the following tests: (1) the R_F values in the *p*-toluenesulphonic acid/*tert.*-pentanol/water mixture were the same, i.e. approximately 0·42 (R_F of cocarboxylase, 0·18; thiamine, 0·7). (2) The molybdate spray of Hanes & Isherwood (1949) gave the same pale-blue colour which slowly increased with time, reaching a maximum in about

Table 2. *Formation of phosphorus compounds on addition of cocarboxylase to a sheep-kidney mitochondrial suspension*

(The incubation mixture contained 0·0125 M pyruvate, 0·0125 M-$NaHCO_3$, 0·0075 M phosphate buffer, pH 7·4, 0·001 M-$MgCl_2$, 0·001 M-ATP, and 2 ml. of a suspension of kidney particles in 0·9% (w/v) KCl; final volume 4 ml.; incubation 60 min. at 40° in O_2.)

Spot no.	R_F	Compound	Phosphorus present: Before incubation (μg.)	Phosphorus present: After incubation (μg.)
1	0·68	Phosphopyruvate	0	134
2	0·54	Inorganic orthophosphate	930	1110
3	0·42	Thiamine monophosphate	0	171
4	0·23	Unknown	0	53
5	0·18	Cocarboxylase	496	175
6	0·09	ATP + ADP	387	59
		Total	1813	1702

Table 3. *Effect of pyruvate on decomposition of cocarboxylase in a sheep-kidney mitochondrial suspension*

(0·01 M-$NaHCO_3$; pyruvate when present, 0·0125 M; phosphate buffer pH 7·4, 0·005 M; other additions as in Table 2; incubation 40 min. at 40°.)

Spot no.	R_F	Compound	Phosphorus present: Before incubation (μg.)	Phosphorus present: After incubation with pyruvate (μg.)	Phosphorus present: After incubation without pyruvate (μg.)
2	0·54	Inorganic orthophosphate	632	659	1248
3	0·42	Thiamine monophosphate	0	Just detectable	65
5	0·18	Cocarboxylase	730	616	387
6	0·09	ATP + ADP	615	316	110
		Total*	1977	1601	1810

* Small amounts of other phosphates, for example AMP, were formed, but these were not estimated.

48 hr. Exposure to ultraviolet light or prolonged heating after spraying shortened the time for maximum colour formation to about 1 hr. (3) Spraying with a solution of potassium bismuth iodide gave a pink-orange colour, not extractable by ether.

By elution with water from several chromatograms, 5 ml. of a solution were obtained, which contained no free orthophosphate but about 100 μg. total phosphorus. This solution and a solution of pure thiamine monophosphate showed the following properties in common: (*a*) No phosphate was liberated on heating for 1 hr. in 2N-H_2SO_4 at 100°. (*b*) No xylene-soluble pigment was formed on treatment with diazotized *p*-aminoacetophenone. (*c*) All the phosphate was liberated on incubation with alkaline phosphatase in 2 hr. at 40° in bicarbonate buffer, pH 8·0. (*d*) Alkaline phosphatase liberated free thiamine which was identified and estimated by coupling with diazotized *p*-aminoacetophenone to give a red, water-insoluble pigment which could be extracted into xylene (Melnick & Field, 1938). (*e*) The amount of thiamine liberated by alkaline phosphatase was about 75% of the amount expected on the basis of the amounts of phosphate released; however, considering that thiamine is unstable at pH 8·0, a deficit is expected.

It may thus be concluded that spot 3 was thiamine monophosphate, and it is referred to as such in the remainder of this paper.

Spot 1, as shown in the following paper, was phosphopyruvate. Spot 4 was unidentified.

In order to measure the changes in the phosphorylated compounds, cocarboxylase was incubated for varying periods (5–40 min.) in a series of Warburg vessels. Each vessel contained 20 μmoles of sodium bicarbonate (0·005M) and 32·4 μmoles of sodium pyruvate (0·0081M). ^{32}P-Labelled orthophosphate was added in order to examine whether the various phosphates incorporated inorganic phosphate. Data on the oxygen uptake, substrate changes, distribution of phosphorus and of radioactivity are shown in Tables 4 and 5. According to

Table 4. *Oxygen uptake, pyruvate consumption and changes in phosphorus-containing substances occurring on incubation of cocarboxylase with sheep-kidney mitochondria*

(Each flask contained initially 20 μmoles of $NaHCO_3$ and 32·4 μmoles of sodium pyruvate; analytical data refer to the total contents of the flask (4·0 ml.).)

Time of incubation (min.)	Vessel no.	O_2 uptake (μmoles)	Pyruvate consumption (μmoles)	Ortho-phosphate present (μmoles)	Cocarboxylase phosphorus present (μmoles)	ATP + ADP phosphorus present (μmoles)	Thiamine monophosphate (spot 3) formed (μmoles)
0	1	0	0	21·2	33·2	8·3	0
5	2	4·15*	3·6	26·2	30·6	3·0	2·0
10	3	8·30*	8·9	24·7	30·3	4·6	2·3
15	4	12·65	14·0	26·8	26·4	4·3	4·2
20	5	15·95	18·1	28·6	22·3	5·8	5·2
25	6	23·60	22·5	28·2	23·6	4·2	5·8
30	7	27·80	25·7	27·2	19·9	5·0	10·0
35	8	26·40	26·6	30·4	20·5	3·2	7·9†
40	9	36·20	27·4	35·3	17·8	3·8	5·0

* O_2 uptake obtained by extrapolation from the average O_2 uptake of vessels 5–9.
† Free thiamine detected on the paper chromatogram at this time.

Table 5. *Incorporation of ^{32}P-labelled orthophosphate into other phosphorus-containing compounds on incubation of cocarboxylase with sheep-kidney mitochondria*

(Same experiment as Table 4)

	Specific radioactivity (counts/min./μg. P) in				
Time of incubation (min.)	Orthophosphate	Cocarboxylase	Adenosine polyphosphates	Labile P of adenosine polyphosphates calculated as all ADP	Thiamine monophosphate
0	1685	0	0	0	0
5	1430	11	411	822	113
10	1340	8	479	958	128
15	1330	20	537	1074	53
20	1290	21	474	948	133
25	1260	35	566	1132	82
30	1170	27	632	1264	61
35	1120	46	432	864	108
40	1080	27	472	944	77

Table 4, the quantity of inorganic orthophosphate progressively increased, whilst that of cocarboxylase progressively decreased. The quantities of ATP + ADP phosphorus fell rapidly within the first 5 min. and then remained fairly constant. Thiamine-monophosphate phosphorus increased to a maximum at 30 min. and then decreased with formation of free thiamine. Table 5 shows that the incorporation of ^{32}P into cocarboxylase was almost negligible. In contrast, incorporation into adenosine polyphosphates was very rapid, but slower than expected (see Krebs, Ruffo, Johnson, Eggleston & Hems, 1953; Bartley, 1953*b*), reaching full equilibrium only after 30 min., instead of 3–4 min. Compared with cocarboxylase, thiamine monophosphate incorporated ^{32}P fairly rapidly, but no equilibrium was reached for reasons that will be discussed later. The correlation of the quantitative aspects (Table 6) shows no consistent relationship between the amount of cocarboxylase broken down and of pyruvate oxidized. These results indicate that added cocarboxylase is not a phosphate carrier in the oxidation of pyruvate.

Metabolism of thiamine and thiamine monophosphate in the presence of pyruvate or fumarate. No formation of thiamine monophosphate could be demonstrated when kidney particles were incubated for up to 40 min. with a range of thiamine concentrations up to 0·005M in the presence of pyruvate or fumarate (0·0125M) and sodium bicarbonate (0·005M). When thiamine was replaced by thiamine monophosphate, no cocarboxylase could be detected by paper chromatography, and about 50% of the added thiamine monophosphate was hydrolysed to free thiamine after 30 min. Spot 1 again appeared. These results suggest that a thiamine phosphorylating enzyme of the type described by Nguyen Van Thoai & Chevillard (1949) in liver and by Steyn-Parvé (1952) in yeast was absent from the suspension, as expected on the basis of the findings of Nielsen & Leuthardt (1950) who showed that the enzymes which phosphorylate thiamine are present in the soluble fraction of rat liver and absent from the mitochondria.

Table 6. *Relationship between cocarboxylase disappearance and pyruvate utilization during the oxidation of pyruvate by sheep-kidney mitochondria*

(The values given are calculated from the data in Table 4.)

Time (min.)	Cocarboxylase removed (μmoles)	Pyruvate removed (μmoles)	Ratio: pyruvate removed / cocarboxylase removed
5	2·6	3·6	1·4
10	2·9	8·9	3·1
15	6·8	14·0	2·1
20	10·9	18·1	1·7
25	9·6	22·5	2·3
30	13·3	25·7	1·9
35	12·7	26·6	2·1
40	15·4	27·4	1·8

Metabolism of cocarboxylase in the presence of α-oxoglutarate and succinate. The sheep-kidney suspensions used in the experiments so far discussed contained up to 0·0125M bicarbonate, this being necessary for the complete oxidation of pyruvate. During incubation the pH of the medium increased from 7·4 to 8·0. To ensure that the results obtained were not due to high pH, pyruvate and bicarbonate were replaced by α-oxoglutarate or succinate. The respiration, substrate consumption and changes in the distribution of phosphorus are given in Table 7 and the specific activities of the phosphorus-containing compounds are given in Table 8. Again, the incorporation of radioactivity into cocarboxylase was very slight and the specific radioactivity of the cocarboxylase was rather smaller with α-oxoglutarate than with succinate, although the oxidation of α-oxoglutarate is known to require cocarboxylase (Barron *et al.* 1941; Stumpf *et al.* 1947). The formation of thiamine monophosphate was not affected by the presence of substrate, but the incorporation of radioactivity into thiamine monophosphate was accelerated by succinate and α-oxoglutarate and was 3–4 times faster than in the presence of pyruvate. With succinate, the incorporation was progressive, with α-oxoglutarate it reached a maximum within 10 min. The breakdown of cocarboxylase was slow when α-oxoglutarate was present, but when the α-oxoglutarate had virtually disappeared (after 20 min.) the amount of cocarboxylase that disappeared was about the same as that which disappeared in the presence of succinate or in the absence of substrate. This parallels the earlier finding (Table 4) that pyruvate diminished the rate of breakdown of cocarboxylase.

Foa, Weinstein, Smith & Greenberg (1952) have shown a similar inhibition of the breakdown of endogenous thiamine phosphates by the addition of insulin, but as no distinction was made between cocarboxylase and thiamine monophosphate it is uncertain whether the dephosphorylation of cocarboxylase or of thiamine monophosphate was inhibited.

In the absence of bicarbonate, thiamine monophosphate was stable. The decomposition in the presence of bicarbonate is probably due to the higher pH. However, after incubation of kidney particles with thiamine monophosphate (up to 0·0025M) at pH 7·4 with α-oxoglutarate or succinate as substrate, and in the absence of bicarbonate, for periods up to 1 hr., no cocarboxylase could be detected by paper chromatography. When radioactive phosphate was added to the incubation medium there was no radioactivity associated with the area of the paper chromatogram where cocarboxylase would be expected.

Table 7. *Metabolism of cocarboxylase in sheep-kidney mitochondria*

(In Expt. 1 the substrate was 57·6 μmoles of α-oxoglutarate; cocarboxylase added approx. 0·002 M. In Expt. 2 substrate was 65·6 μmoles of succinate; cocarboxylase added approx. 0·00175 M. In Expt. 3 no substrate was added; cocarboxylase added approx. 0·0015 M. Other additions as in Table 2. The analytical data refer to the total content of each Warburg vessel (4 ml.).)

Expt. no.	Substrate added	Time of incubation (min.)	O_2 uptake (μmoles)	Quantity of added substrate removed (μmoles)	Ortho-phosphate found (μmoles)	Cocarboxylase phosphorus found (μmoles)	ATP + ADP phosphorus found (μmoles)	Thiamine monophosphate phosphorus found (μmoles)
1	α-Oxo-glutarate	0	0·0	0·0	18·2	16·9	8·30	0·00
		10	24·8	40·2	17·4	16·0	5·00	1·61
		20	51·1	55·4	21·0	13·1	5·64	3·02
		30	84·3	56·1	16·6	7·7	5·58	2·15
2	Succinate	0	0·0	0·0	19·2	13·5	7·8	0·00
		10	25·6	45·8	19·2	8·9	2·8	2·03
		20	58·3	55·4	17·4	7·4	4·4	3·35
		30	80·9	54·7	14·9	6·5	4·6	4·14
3	None	0	0·0	0·0	16·0	11·6	7·3	0·00
		10	2·1	0·0	22·2	7·0	0·83	2·11
		20	2·8	0·0	25·6	4·8	0·00	2·92
		30	3·3	0·0	26·1	3·3	0·00	4·39

Table 8. *Incorporation of ^{32}P-labelled orthophosphate into other phosphorus-containing compounds on incubation of cocarboyxlase with sheep-kidney mitochondria*

(Same experiments as described in Table 7.)

			Specific radioactivity (counts/min./μg. P) in				
Expt. no.	Substrate added	Time of incubation (min.)	Orthophosphate	Cocarboxylase	Adenosine polyphosphates	Labile P of adenosine polyphosphates calculated as all ADP	Thiamine monophosphate
1	α-Oxoglutarate	0	2088	0	0	0	0
		10	1630	5	683	1366	442
		20	1265	5	525	1050	305
		30	1235	35	662	1324	452
2	Succinate	0	1655	0	0	0	0
		10	1211	12	389	778	95
		20	1150	20	428	956	314
		30	1295	77	666	1332	633
3	None	0	1546	0	0	0	0
		10	1022	10	99	198	65
		20	894	12	0	0	58
		30	836	40	0	0	—

Table 9. *Incorporation of ^{32}P-labelled orthophosphate into thiamine monophosphate*

(Each vessel contained 6·9 μmoles of thiamine monophosphate, 55·7 μmoles of α-oxoglutarate and about 70 mg. dry wt. of kidney particles; other additions as in Table 2.)

Time of incubation (min.)	Ratio: $\frac{A^*}{B} \times 100$.
0	0·0
5	5·4
10	7·5
15	12·0
20	13·3
30	16·5
60	35·0

* $\frac{A}{B} = \frac{\text{specific activity of thiamine monophosphate}}{\text{specific activity of orthophosphate}}$.

It was thought that cocarboxylase might not become radioactive because it might not penetrate into the mitochondria. However, this assumption was not borne out by direct measurement of the distribution of cocarboxylase between mitochondria and medium by the methods described by Bartley & Davies (1954). This gave a ratio of the cocarboxylase in the mitochondria/the cocarboxylase in the medium of 0·8 after 10 min. at 20°.

Turnover of thiamine monophosphate. The rate of incorporation of ^{32}P-labelled orthophosphate into thiamine monophosphate was measured at pH 7·4 with α-oxoglutarate as the substrate. Under these conditions the concentration of thiamine monophosphate was found to remain constant. The rate of incorporation of radioactivity into thiamine monophosphate (Table 9) shows that ^{32}P is incorporated

at a slow but steady rate of about 0·6 % (0·053 μ-moles)/min.

It has already been shown that the kidney suspensions do not phosphorylate thiamine when pyruvate plus bicarbonate was the substrate, and that added thiamine monophosphate is dephosphorylated. Phosphorylation of thiamine was also tested under conditions where thiamine monophosphate was stable (pH 7·4 with α-oxoglutarate as substrate) but no evidence of phosphorylation was found. The radioactivity of the thiamine monophosphate area found after 1 hr. indicated less than 4 μg. thiamine monophosphate compared with the exchange rate of added thiamine monophosphate of about 80 μg./hr. Thus, thiamine is not appreciably phosphorylated in the mitochondria under the conditions tested.

DISCUSSION

Incorporation of ^{32}P-labelled orthophosphate into cocarboxylase. Although incubation of respiring mitochondria with cocarboxylase and ^{32}P-labelled orthophosphate leads to the appearance of the labelled phosphorus in cocarboxylase, the incorporation is very slight and bears no constant relationship to the extent of substrate oxidation (Table 10). It is therefore very unlikely that cocarboxylase acts as a phosphate carrier. Kiessling & Lindahl (1952) have already claimed to have demonstrated the incorporation of ^{32}P into cocarboxylase in mitochondrial suspensions containing $KH_2{}^{32}PO_4$, but it is uncertain whether the isolated cocarboxylase contained thiamine monophosphate, a point raised by the results reported in this paper. Kiessling & Lindahl also report a 'fair' proportionality between the rate of incorporation and of oxygen consumption when pyruvate was the substrate. This does not hold when different ketonic acids are compared (Table 10).

Route of formation of thiamine monophosphate. The fact that the specific activity of thiamine monophosphate always remained below that of the labile P of ATP and inorganic P could be accounted for by the assumption that thiamine monophosphate can be formed from cocarboxylase by more than one route. If it were formed only by dephosphorylation of the cocarboxylase to thiamine and rephosphorylation to the monophosphate, it would have at least the same specific activity as the ATP or inorganic P at the same time (whichever was the lower). Thiamine monophosphate formed directly by the hydrolysis of one phosphate group of cocarboxylase cannot be radioactive. The occurrence of these two reactions could account for the observed specific activities of the thiamine monophosphate, but as thiamine was not phosphorylated by the preparations used, thiamine monophosphate cannot be formed by a route involving free thiamine, and the above explanation therefore cannot be correct. However, the facts are in agreement with the assumption that thiamine is rephosphorylated in some combined form. Reed & DeBusk (1952) have shown that 'lipothiamide pyrophosphate' is the active form of cocarboxylase for some strains of *Escherichia coli*. If this holds also for animal tissues, then its formation must readily occur because cocarboxylase can fully re-activate the cocarboxy-

Table 10. *Relations between the amounts of phosphorus of cocarboxylase exchanged and the amounts of substrate oxidized*

(Phosphorus exchange calculated from the data given in Tables 5 and 8.)

Substrate	Incubation time (min.)	Cocarboxylase P exchanged (μmoles)	Substrate removed (μmoles)	Ratio: substrate removed/ cocarboxylase P exchanged
Pyruvate	5	0·254	3·6	14·5
	10	0·198	8·9	44·9
	15	0·500	14·0	28·0
	20	0·540	18·1	33·5
	25	0·923	22·5	24·4
	30	0·767	25·7	33·4
	35	1·36	26·6	19·5
	40	0·831	27·4	33·0
α-Oxoglutarate	10	0·0518	40·2	776
	20	0·0669	55·4	832
	30	0·442	56·1	127
Succinate	10	0·134	45·8	342
	20	0·235	55·4	235
	30	0·800	54·7	68·4
None	10	0·114	—	—
	20	0·156	—	—
	30	0·767	—	—

lase-free purified pyruvate oxidase system (Jagannathan & Schweet, 1952). The pyruvate and α-oxoglutarate oxidase preparations of animal tissues have been shown to contain α-lipoic acid (Schweet & Cheslock, 1952; Sanadi, Littlefield & Bock, 1952). If lipothiamide pyrophosphate was dephosphorylated to lipothiamide and rephosphorylated to the monophosphate, with ATP or inorganic P serving as the source of phosphate, then the subsequent hydrolysis of the amide linkage would result in the formation of radioactive thiamine monophosphate. The reactions postulated are as follows:

(1) cocarboxylase + α-lipoic acid → lipothiamide pyrophosphate;

(2) lipothiamide pyrophosphate → lipothiamide + 2 orthophosphate;

(3) lipothiamide + orthophosphate or ATP → lipothiamide monophosphate;

(4) lipothiamide monophosphate → α-lipoic acid + thiamine monophosphate.

The incorporation of radioactivity into added thiamine monophosphate would be accounted for if reaction (4) occurred in the reverse direction (probably by a different route), but at a much slower rate than the overall rate of reactions (1)–(4), so that the balance of the reactions would favour the formation of free thiamine monophosphate.

SUMMARY

1. The behaviour of thiamine, thiamine monophosphate, cocarboxylase, thiamine triphosphate, oxythiamine, and oxythiamine diphosphate on paper chromatograms is described; *p*-toluenesulphonic acid/*tert.*-pentanol/water was the solvent. The substances are located by spraying with potassium bismuth iodide.

2. Of the thiamine derivatives tested at 0·005 M (thiamine, thiamine monophosphate, cocarboxylase and thiamine triphosphate), only thiamine triphosphate affected the oxidation of pyruvate (40 % inhibition) by sheep-kidney particles; none altered the ratio of the oxygen uptake/pyruvate consumed.

3. Cocarboxylase is converted into thiamine monophosphate by washed suspensions of kidney particles. The amount of cocarboxylase disappearing is less in the presence of pyruvate or α-oxoglutarate than in the absence of substrate, or in the presence of succinate.

4. On incubation of cocarboxylase and ^{32}P-labelled orthophosphate with respiring kidney particles, a slow incorporation of radioactivity into the cocarboxylase occurred. This was unrelated to the amount of substrate oxidized.

5. Thiamine monophosphate formed by breakdown of cocarboxylase in the presence of ^{32}P-labelled orthophosphate was much more radioactive when the tissue suspension contained oxidizable substrate than in the absence of substrate; α-oxoglutarate caused a more rapid incorporation of radioactivity than did succinate.

6. The tissue suspensions were unable to synthesize cocarboxylase from thiamine monophosphate or thiamine, or to synthesize thiamine monophosphate from thiamine; thus it is unlikely that the radioactivity found in thiamine monophosphate was incorporated by rephosphorylation of free thiamine. A mechanism of phosphorylation, assuming that 'lipothiamide' is the reactive form of thiamine is put forward.

7. The steady-state exchange of the phosphate of thiamine monophosphate during the oxidation of α-oxoglutarate has been measured as 0·053 μmole/min.

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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; *iso*propyl 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 0·0025 M 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 1 N-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.