CCXLV. PYRUVATE OXIDATION IN BRAIN

VII. SOME DIALYSABLE COMPONENTS OF THE PYRUVATE OXIDATION SYSTEM¹

BY ILONA BANGA, SEVERO OCHOA AND
RUDOLPH ALBERT PETERS

From the Department of Biochemistry, Oxford

(Received 1 November 1939)

By the use of finely ground preparations (dispersions) of pigeon's brain in phosphate buffer, we have shown in a previous paper [Banga *et al.* 1939, 1] that cocarboxylase (vitamin B₁ pyrophosphate) is the active form of vitamin B₁ concerned with the oxidation of pyruvate in brain, and, further, that C₄ dicarboxylic acids activate this oxidation in a catalytic manner. The action of succinate on the oxidative decarboxylation of pyruvate by washed muscle preparations had been observed shortly before by Annau & Erdös [1939]. We shall show in this paper that, upon dialysing the brain dispersions, not only does the effect of the C₄ dicarboxylic acids become more definite, but the need for other substances in the oxidation system can also be demonstrated. This is the case with inorganic phosphate and "adenine nucleotide" (this term being here used to include adenylic acid and adenosine triphosphate). The necessity of "pyridine nucleotide" (cozymase) has been made likely. Further, it has been recently shown by Ochoa [1939] that magnesium (or manganese) ions are also indispensable components of the oxidation system. We now know that at least the following substances are components of the pyruvate oxidation system of brain and, probably, of other animal tissues: (1) cocarboxylase, (2) inorganic phosphate, (3) C₄ dicarboxylic acids (succinate, fumarate, etc.), (4) "adenine nucleotide", (5) Mg⁺⁺ (or Mn⁺⁺) and, probably, (6) cozymase (pyridine nucleotide).

It will further be shown that citrate or other intermediates of the citric acid cycle of Krebs & Johnson [1937], such as α -ketoglutarate, are much less active than C₄ dicarboxylic acids in the pyruvate oxidation system of brain, thus making it very unlikely that oxidation of pyruvate in brain can take place through such a cycle.

In another section the oxidation of phosphoglyceric acid by brain dispersions is studied. Phosphoglyceric acid is rapidly converted into pyruvic acid by the dispersions and is, therefore, readily oxidized. It has been found that the reactions

Phosphoglyceric acid $\rightleftharpoons$ Phosphopyruvic acid,

Phosphopyruvic acid + Adenylic acid $\rightarrow$ Pyruvic acid + Adenosine polyphosphate,
take place in brain in the same way as in muscle.

Experimental methods

Brain dispersions were prepared by thoroughly grinding, in an ice-cold mortar, 1 part of tissue with 4 parts of ice-cold 0.9% KCl; the mixture was then pressed through muslin. The dispersion was dialysed for various periods, under

¹ Preliminary report, Banga *et al.* [1939, 2].

constant mechanical shaking, in collodion tubes of narrow bore (taking about 10 ml. each) against 3 litres of 0.4% KCl, the temperature of which was kept between 1 and 3°. Unless otherwise stated the respiration experiments were carried out with 1.5 ml. samples of dialysed dispersion (equivalent to about 300 mg. brain) made up with additions (dissolved in water) to 2 ml. and buffered to pH 7.3. Both Barcroft and Warburg manometers have been used with air¹ in the gas space. Temperature 38° or 28°. Respiration experiments were mostly carried out in duplicate. The various additions were measured into the main space of the manometer bottles (best placed in ice) before the enzyme which was always added last. Readings were started after 5 min. temperature equilibration.

Pyruvate was determined colorimetrically by the salicylaldehyde method of Straub [1936]; the colorimetry was carried out against a freshly prepared standard of pure Na pyruvate using a Leitz compensation colorimeter.

Phosphate was determined colorimetrically by the method of Lohmann & Jendrassik [1926] and the pyrophosphate, phosphopyruvate and phosphoglycerate P fractions, by 7 min. hydrolysis at 100° in *N* HCl, hydrolysis in alkaline I₂, and difference between ashing and 180 min. hydrolysis in *N* HCl at 100° respectively.

Preparations

Freshly made up solutions of crystalline Na pyruvate were employed as usual.

Ba phosphoglycerate. Prepared from yeast by the method of Vercellone & Neuberger [1935]. Used as Na salt after decomposition with Na₂SO₄.

α-Ketoglutaric acid. A pure specimen prepared by Mr C. Long in this Laboratory.

Na oxaloacetate. A pure specimen from the Biochemical Laboratory of Szeged, Hungary.

Cozymase (90% pure). Prepared by Mr L. A. Stocken in this Laboratory by the method of Meyerhof & Ohlmeyer [1937].

Ba adenosinetriphosphate. A specimen from muscle kindly supplied by Prof. O. Meyerhof. Used as Na salt after decomposition with Na₂SO₄.

All other substances were commercial preparations including cocarboxylase (kindly presented by Messrs Merck and Co., U.S.A.) and adenylic acid (specimens from Messrs Fraenkel and Landau and Dr G. Henning).

All the acids were used as the Na salts.

We are much indebted to those mentioned for their help and presents.

1. EXPERIMENTS WITH PIGEON'S BRAIN

A. Effect of phosphate, fumarate and "adenine nucleotide" on the oxidation of pyruvate

After short periods of dialysis (2-3 hr.) the capacity of brain dispersions to oxidize added pyruvate is very small but their activity can be fully restored to the original value by addition of inorganic phosphate, fumarate and adenylic acid (or adenosinetriphosphate). To produce full reactivation the addition of all three substances is indispensable; if one of them is not added, pyruvate is no better oxidized than in the absence of all three. These facts are shown in Fig. 1, which gives in synthesis the results of many experiments of this type. Table I shows that adenylic acid can be replaced by adenosinetriphosphate.

¹ Pure oxygen was not better than air.

Table I. *Effect of adenosinetriphosphate (ATP) on the oxidation of pyruvate by dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed 2 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.01 M), fumarate (0.005 M), and pyruvate (0.0091 M). Air, 38°.

Time	μ l. O_2 uptake with		
	No addition	Adenylic acid (0.0014 M)	ATP (0.00035 M)
10 min.	55	186	186
20 min.	91	290	315

Fig. 1. Effect of phosphate (0.05 M), fumarate (0.005 M) and adenylic acid (0.00014 M) on the oxidation of pyruvate (0.0091 M) by dialysed dispersions from pigeon's brain. 1.5 ml. enzyme (dialysed 2 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$). The complete system contains enzyme + Mg^{++} + phosphate (P) + fumarate (fum.) + adenylic acid (Ad.) + pyruvate (pyr.). Sample with no phosphate buffered with $NaHCO_3$ to pH 7.3. Air, 38°.

If brains from B_1 -avitaminous pigeons (showing symptoms) are used, and the dispersions prepared from them dialysed as usual, cocarboxylase must also be added to obtain reactivation of the pyruvate oxidation system. In fact, in order to obtain marked activation by cocarboxylase, phosphate, fumarate and adenylic acid must all be added. The experiments of Table II show this for fumarate. The effect of cocarboxylase is small if only pyruvate, phosphate and adenylic acid are present but of the same order as before dialysis [cf. Banga *et al.* 1939, 1], if fumarate is also added.

That phosphate, fumarate and adenylic acid are necessary for the oxidation of pyruvate, and are not merely stimulating the oxidation of residual substrate which might be still present in the dispersions is clearly shown (1) by the fact (cf. Fig. 1) that the excess O_2 uptake of the complete system over the O_2 uptake of the samples containing everything but pyruvate is about three times larger than that of these samples, and (2) by the larger amounts of pyruvate disappearing when all the three substances are added to the dialysed dispersions (cf. Table III).

Table II. *Effect of cocarboxylase on the oxidation of pyruvate by dialysed dispersions from avitaminous pigeon's brain*

1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 M), and adenylic acid (0.00028 M). Air, 38°.

Exp.*	Cocarboxylase $\mu g.$	$\mu l.$ O_2 uptake in 20 min. with			
		No addition	Fumarate (0.005 M)	Pyruvate (0.0091 M)	Fumarate (0.005 M) + pyruvate (0.0091 M)
1	0.0	26	33	70	75
	0.5	57	74	93	215
	Increase due to cocarboxylase	+31	+43	+23	+140
2	0.0	42	78	82	159
	0.7	45	84	120	261
	Increase due to cocarboxylase	+3	+6	+38	+102

* 2 out of 4 experiments quoted.

Table III. *Effect of phosphate, fumarate and adenylic acid on the aerobic disappearance of pyruvate in dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed 2 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$) and pyruvate (0.0182 or 0.0091 M). Air, 38°.

Exp.	Phosphate (mol. $\times 10^{-3}$)	Fumarate (mol. $\times 10^{-3}$)	Adenylic acid (mol. $\times 10^{-3}$)	Pyruvic acid found after 20 min. mg.
1	—	5	0.14	3.65
	20	5	0.14	2.98
	50	5	0.14	2.85
	100	5	0.14	2.55
2	50	5	—	1.71
	50	—	0.14	1.42
	50	5	0.14	1.12
3	—	5	0.14	1.90
	50	—	0.14	1.50
	50	5	0.14	1.07

Fig. 2. Pyruvate oxidation in relation to the concentration of inorganic phosphate. O_2 uptake in 20 min. plotted against the reciprocal logarithms of the molar concentrations of phosphate. 1.5 ml. enzyme (pigeon's brain dispersion dialysed 2 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), fumarate (0.005 M), adenylic acid (0.00014 M), pyruvate (0.0091 M) and various concentrations of phosphate ($Na^+ - K^+$, pH 7.3). Air, 38°.

The effect of adenine nucleotide is obtained with catalytic concentrations. That the effect of fumarate and other C_4 dicarboxylic acids, although requiring rather larger concentrations to be optimum, is also a catalytic one, will be shown in the next section.

Optimum O_2 uptakes, on the other hand, require high concentrations of phosphate (0.05 to 0.1 M). The continuous line of Fig. 2 shows the relation between the O_2 uptake, in the presence of pyruvate, and phosphate concentration. After 2 hr. dialysis there is still an appreciable amount of phosphate present in the brain dispersions. The average value of several determinations corresponds to about 0.001 M phosphate (in 1.5 ml. dispersion diluted to 2 ml.) so that the point in Fig. 2 for 0.001 M phosphate corresponds to samples with no phosphate added (buffered with $NaHCO_3$ to pH 7.3). The extrapolation to zero (dotted line) is merely tentative, but it does not seem unreasonable to suppose that, at concentrations of phosphate much below 0.001 M , the O_2 uptake would be further significantly reduced.

B. Comparison of the catalytic actions of C_4 dicarboxylic, α -ketoglutaric and citric acids on pyruvate oxidation

The net O_2 uptake of pyruvate in dialysed dispersions is always much greater in the presence of a C_4 dicarboxylic acid than in its absence; in fact, it is more than doubled in most cases (Table IV). The effects of succinate, fumarate and malate are practically identical. Smaller effects were obtained with oxaloacetate in one experiment, but this is probably due to the fact that part of it is decarboxylated to pyruvate with a consequent decrease in its optimum concentration.

The pronounced effect of the C_4 dicarboxylic acids is in marked contrast with the small activity of citrate. In Exp. 1 (Table IV), citrate had no effect whatever on the O_2 uptake of pyruvate, and in Exp. 2, 0.005 M citrate increased the net O_2 uptake of pyruvate by 36 μ l. (from 110 to 146 μ l.) or +33 %, whereas the same concentration of fumarate produced the almost four times greater rise of 131 μ l. (from 110 to 241 μ l.) or +120 %. In fact, citrate itself is not significantly oxidized by the brain dispersions.¹ As regards brei, this fact had already been observed by Long *et al.* [1939].

α -Ketoglutarate has also a smaller effect than the C_4 dicarboxylic acids on the oxidation of pyruvate by the dialysed brain dispersions (cf. Table IV, Exps. 4 and 7). Thus in Exp. 4, 0.005 M α -ketoglutarate increased the net O_2 uptake of pyruvate by 53 μ l. (from 122 to 175 μ l.) or +43 %, whereas the same concentration of fumarate increased it by 143 μ l. (from 122 to 265 μ l.) or +118 %. In Exp. 7 the effect of α -ketoglutarate is significantly greater than in Exp. 4, but also much smaller than that of fumarate. In the absence of pyruvate, α -ketoglutarate causes a significant increase of the O_2 uptake [cf. McGowan & Peters, 1937, for experiments with brain brei].

C. "Adenine nucleotide" and the molar oxygen/pyruvate ratio

The ratio of O_2 taken up to pyruvate disappearing was determined by McGowan [1937] using washed pigeon's brain brei. Whereas the theoretical value for complete oxidation of pyruvate according to the reaction

¹ In Exp. 1 (Table IV), 0.015 M citrate was added to the sample without pyruvate.

Table IV. *Comparison of the catalytic actions of C₄ dicarboxylic, α-ketoglutaric and citric acids on the oxidation of pyruvate by dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed for various periods) to 2 ml. with additions including 0.1 mg. Mg⁺⁺ (as MgCl₂), phosphate (0.05 M), adenylic acid (0.00028-0.00014 M), and pyruvate (0.0091 M). Air, 38°.

Exp.	Duration of dialysis hr.	Sample	No addition	μl. O ₂ uptake in 20 min. with					
				Succinate (0.007 M)	Fumarate (0.005 M)	Malate (0.005 M)	Oxaloacetate (0.005 M)	α-Ketoglutarate (0.005 M)	Citrate (0.005 M)
1	0.5	Enzyme	140	—	—	—	—	—	150*
		Enzyme + pyruvate	272	—	—	—	—	—	270
		Net O ₂ uptake of pyruvate	132	—	437	—	—	—	120 (0.0)
2	2.5	Enzyme	36	—	—	—	—	—	40
		Enzyme + pyruvate	146	—	81	—	—	—	186
		Net O ₂ uptake of pyruvate	110	—	241 (+120%)	—	—	—	146 (+33%)
3	2	Enzyme	34	—	—	81	80	—	—
		Enzyme + pyruvate	147	—	—	296	277	—	—
		Net O ₂ uptake of pyruvate	113	—	—	215 (+90%)	197 (+75%)	—	—
4	3	Enzyme	20	—	80	—	—	180	—
		Enzyme + pyruvate	142	—	345	—	—	355	—
		Net O ₂ uptake of pyruvate	122	—	265 (+118%)	—	—	175 (+43%)	—
5	3.5	Enzyme	25	67	—	—	—	—	—
		Enzyme + pyruvate	107	262	—	—	—	—	—
		Net O ₂ uptake of pyruvate	82	195 (+138%)	—	—	—	—	—
6†	3	Enzyme	27	57	59	—	—	—	—
		Enzyme + pyruvate	72	208	198	—	—	—	—
		Net O ₂ uptake of pyruvate	45	151 (+235%)	139 (+210%)	—	—	—	—
7‡	3	Enzyme	44	—	82	—	—	103§	—
		Enzyme + pyruvate	120	—	262	—	—	228§	—
		Net O ₂ uptake of pyruvate	76	—	180 (+137%)	—	—	125 (+65%)	—

* Citrate 0.015 M.

† Dispersion from B₁-avitaminous brain; all samples with coenzyme added.

‡ Dispersion from B₁-avitaminous brain; all samples with coenzyme added; also coenzyme (0.00004 M).

§ α-Ketoglutarate 0.0035 M. Figures in brackets give the percentage increase in the net O₂ uptake of pyruvate due to the various acids compared.

is 2.5, McGowan found, as average of a number of experiments at 38°, 1.76, with extreme values of 1.60 and 1.98, indicating that the oxidation was not complete. Later Long [1938], also working with washed brain brei at 38°, found that production of acetic acid, and, to a much less extent, of lactic acid, practically accounted for the whole of the pyruvate not completely oxidized.

We have determined the O_2 /pyruvate ratio, in dialysed dispersions, in experiments at 38° and 28°, for respiration periods of 20 min. This short period was chosen because, at 38°, the rate of O_2 uptake in the dispersions (whether undialysed or dialysed), in the presence of pyruvate and all necessary additions, falls off very rapidly after 20–30 min., and becomes very small later. In fact, at 38°, the rate of O_2 uptake never remains constant over any reasonable length of time. Conditions are better at 28°; at this temperature, the rate of O_2 uptake, in the presence of pyruvate, remains constant for 40 min. and falls off markedly after this time (cf. Fig. 3). Some enzymic component of the oxidation system seems to be very unstable in the dispersions and is certainly highly sensitive towards higher temperatures.

The ratio mol. O_2 /mol. pyruvate in dialysed dispersions at 38°, has been found to be about 1.2, roughly half the theoretical value for complete oxidation, and approximately two-thirds of McGowan's value. At 28°, however, the ratio is about 1.5 which is much nearer to the values obtained with brain brei. The higher instability of some enzymic component of the system in the dispersions as compared with brei, referred to above, is probably responsible for the even less complete degree of oxidation of pyruvate in the former.

However, what is especially interesting here is the fact that the degree of oxidation of pyruvate by the dialysed dispersions is, in the absence of adenylic acid, much lower than in its presence. In fact, without adenylic acid, not only are both the O_2 uptake and the pyruvate disappearance much smaller but the ratio mol. O_2 /mol. pyruvate decreases to nearly 0.5, which is the theoretical value for the oxidative decarboxylation of pyruvate according to the reaction

This indicates that "adenine nucleotide" is also necessary for the oxidation of pyruvate beyond the stage of oxidative decarboxylation.

The experiments of Table V show that, under our experimental conditions, adenylic acid doubles the value of the O_2 /pyruvate ratio.

The two experiments at 38° of Table V have been carried out in duplicate, the experiment at 28°, with no adenylic acid added, in quadruplicate, and the

Fig. 3. Effect of adenylic acid on the oxidation of pyruvate by dialysed dispersions from pigeon's brain at 28°. 1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions. The complete system (curve I) contains enzyme, 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 M), fumarate (0.005 M), adenylic acid (0.00014 M) and pyruvate (0.0091 M). Curve II: no adenylic acid. Curve III: no pyruvate. Curve IV: no pyruvate, no adenylic acid. Air, 28°.

two experiments at 28°, with adenylic acid, in triplicate, both as regards measurements of O₂ uptake and pyruvate estimations. The results in the table are average values, the individual values varying not more than $\pm 5\%$.

The experiments were carried out by placing 0.2 ml. of 25% trichloroacetic acid in the side bulbs of the Warburg bottles and stopping the reaction at the desired time by tipping in the trichloroacetic acid. For the zero time values of pyruvate¹ (both in experiments without or with adenylic acid) the trichloroacetic acid solution was tipped in after 5 min. temperature equilibration in the bath, immediately after taking the first reading of the other manometers. In the experiment at 28°, without adenylic acid, as the pyruvate disappearance in 20 min. would be very small and, therefore, difficult to estimate accurately, the respiration was continued for 1 hr. and the final pyruvate value determined at the end of this period. During this time the O₂ uptake increases linearly with time (cf. Fig. 3, curve II), in other words, the respiration rate is constant. The rate of pyruvate disappearance must have also been constant and the ratio O₂/pyruvate after 20 min. must have been the same as after 60 min. The values for the pyruvate disappearance in 20 min. given in Table V, have been calculated on this assumption.

Table V. *Effect of adenylic acid on the O₂/pyruvate ratio in dialysed dispersions from pigeon's brain at 38° and 28°*

1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions including 0.1 mg. Mg⁺⁺ (as MgCl₂), phosphate (0.05 M), fumarate (0.005 M), and pyruvate (0.0091 M). Air.

Temp.	Adenylic acid (mol. $\times 10^{-3}$)	Extra O ₂ uptake due to pyruvic acid in 20 min.		Pyruvic acid disappeared in 20 min.		mol. O ₂ mol. pyruvic acid
		μ l.	μ mol.	mg.	μ mol.	
38°	—	43	1.920	0.342	3.880	0.495
	0.14	215	9.580	0.698	7.930	1.21
38°	—	47	2.100	0.270	3.070	0.68
	0.14	254	11.400	0.910	10.410	1.10
28°	—	15	0.656	0.077	0.873	0.75
	0.14	157	7.000	0.436	4.950	1.42
	0.14	131	5.850	0.348	3.960	1.48

After removing the protein precipitate by centrifuging the pyruvate was determined on 1 ml. aliquots of the clear filtrate, as described in the section on experimental methods.

The net O₂ uptake of pyruvate was always determined as the difference between control samples and those with added pyruvate, either with or without adenylic acid.

Table VI. *O₂/pyruvate ratio of the extra metabolism produced by adenylic acid in the experiments at 28° of Table IV*

Extra O ₂ uptake caused by adenylic acid in 20 min.		Extra pyruvic acid disappearance caused by adenylic acid in 20 min.		mol. O ₂ mol. pyruvic acid
μ l.	μ mol.	mg.	μ mol.	
177	7.890	0.349	3.970	1.99
139	6.200	0.246	2.790	2.22

In the experiments at 28°, the extra O₂ uptake (with pyruvate) and the extra disappearance of pyruvate caused by adenylic acid were also determined.

¹ Always duplicates at least

In this way the O_2 /pyruvate ratio of the excess metabolism produced by adenylic acid can be estimated. As shown in Table VI this ratio is considerably nearer the theoretical value of 2.5 for complete oxidation. This means that the extra pyruvate disappearing under the action of adenylic acid is being much more completely oxidized to CO_2 and H_2O .

D. Effect of cozymase

As already reported [Banga *et al.* 1939, 2] addition of cozymase to samples containing 0.00014 *M* adenylic acid has little or no effect when the enzyme has been dialysed for periods of 2–3 hr., but causes significant increases of the O_2 uptake after longer periods of dialysis. Since then it has been found that, on the one hand, cozymase (90 % purity) can completely replace adenylic acid. This is the case (with pigeon's brain dispersions) even after 15 hr. dialysis (Table VII).

Table VII. *Effect of cozymase on the oxidation of pyruvate by dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed for various periods) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 *M*), fumarate (0.005 *M*), and pyruvate (0.0091 *M*). Air, 38°.

Exp.	Duration of dialysis hr.	Adenylic acid (mol. $\times 10^{-3}$)	Cozymase (mol. $\times 10^{-3}$)	$\mu l.$ O_2 uptake in 20 min.
1	8	—	—	71
		0.14	—	130
		2.80	—	131
		0.14	0.14	190
2	3	—	—	95
		0.14	—	235
		0.28	—	243
		—	0.14	343
		—	0.28	390
3	7.5	—	—	61
		1.4	—	250
		—	0.28	270
		1.4	0.28	287
4	6	0.14	—	136
		1.4	—	230
		—	0.14	180
		1.4	0.14	240
5	15	—	—	57
		0.07	—	84
		0.14	—	103
		0.07	0.035	118
		0.28	—	172
		—	0.28	203
		0.14	0.07	180
		0.14	0.14	187
		0.70	—	188
		0.14	0.21	198

On the whole cozymase is, molecule for molecule, more active than adenylic acid (Table VII, Exps. 2, 4, 5). On the other hand, after periods of dialysis of 6 hr. or over, it frequently happens that increase of the adenylic acid beyond 0.00014 *M* produces a marked increase of the O_2 uptake (Table VII, Exp. 4), when further addition of cozymase has little, if any, effect. Occasionally, however, the higher concentrations of adenylic acid do not give rise to increased respiration which can then be obtained with cozymase (Table VII, Exp. 1).

In view of these fresh facts the question as to whether cozymase is indispensable in the pyruvate oxidation system of brain obviously cannot be solved

unless the brain dispersions could be submitted to much longer periods of dialysis without irreversible inactivation of the enzyme proteins. Recently we have been able to dialyse the brain dispersions for periods over 15 hr., up to 24 hr., after which time the activity of the enzyme, after addition of Mg^{++} , phosphate, fumarate and adenylic acid, is only about 25% of the original. Even then (with 0.0007 *M* adenylic acid) cozymase produced little effect. In this connexion we might recall that Meyerhof & Ohlmeyer [1936; 1937], using acetone powder extracts from muscle, obtained full reactivation of the lactic acid formation, in the partial reaction triosephosphate + pyruvate, after 15 hr. dialysis, by adding Mg^{++} and adenylic acid. Only after 36–48 hr. dialysis was addition of cozymase indispensable and, even so, less cozymase was required to produce a certain degree of reactivation when adenylic acid was present. They point out that, in order to prove the indispensability of cozymase in their system, the cozymase had to be reduced to under 1/1000 of the amount originally present in the enzyme preparations. When this was attained, however, the capacity of the extracts to form lactic acid from glycogen was irreversibly lost.

On the whole our experiments seem to lend some support to the view that cozymase is another component of the pyruvate oxidation system of brain. In addition, if the reaction oxaloacetate $\rightleftharpoons$ malate is playing a catalytic part here, as it does in the oxidation of triosephosphate in muscle [Szent-Györgyi, 1937], the necessity of cozymase is evident.

Another question is how can cozymase replace adenine nucleotide. Either it can function as cophosphorylase [cf. Ohlmeyer & Ochoa, 1937] or it is broken down to some extent by the brain dispersions with formation of cophosphorylase ("adenine nucleotide"). Experiments on the liberation of inorganic phosphate from phosphoglyceric acid, to be reported in the next section, would seem to favour the latter view.

E. Oxidation of phosphoglycerate

If phosphoglyceric acid is used as substrate, the O_2 uptake with dialysed brain dispersions, after addition of Mg^{++} , phosphate, fumarate and adenylic acid, is not much lower than with pyruvic acid (Table VIII). In the two experiments of Table VIII (out of several similar experiments), the average net O_2

Table VIII. *Oxidation of phosphoglyceric acid by dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 *M*), fumarate (0.005 *M*) and adenylic acid (0.00028 *M*). Air, 38°.

Exp.	NaF <i>M</i>	Pyruvate <i>M</i>	Phosphoglycerate <i>M</i>	μ l. O_2 uptake in 20 min.
1	—	—	—	70
	0.04	—	—	43
	—	0.0091	—	348
	0.04	0.0091	—	242
	—	—	0.018	257
	0.04	—	0.018	40
2	—	—	—	65
	—	0.0091	—	310
	—	—	0.018	270

uptake of pyruvate in 20 min. was 260 μ l., and that of phosphoglycerate 196 μ l., or 75% of the former. The observation of Jowett & Quastel [1937], that phosphoglycerate increased but slightly the O_2 uptake of guinea-pig brain slices, must

have been due to poor penetration of the compound. Similar negative results (from 1935 onwards) have repeatedly been obtained in this laboratory with pigeon's brain brei using either phosphoglyceric or phosphopyruvic acid. This recalls the failure to obtain catatorulin effects with small amounts of cocarboxylase in brain brei or slices, whereas they are easily obtained in dispersions [cf. Banga *et al.* 1939, 1].

Phosphoglyceric acid is not directly oxidized. If its conversion into phosphopyruvic acid is prevented by NaF, it produces no increase whatever of the O_2 uptake (Table VIII, Exp. 1); under identical conditions the oxidation of pyruvate is only inhibited by 40% [cf. Peters *et al.* 1935]. In fact, the oxidation of phosphoglycerate is due to its rapid conversion into pyruvate¹ and inorganic phosphate.² This conversion had been observed to take place with rabbit's brain extracts by Mazza & Valeri [1935]. The additional points in Table IX are the

Table IX. *Liberation of inorganic phosphate from phosphoglyceric acid in dialysed dispersions from pigeon's brain*

1 ml. enzyme (dialysed 6 hr.) to 1.5 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.0066 *M*), and phosphoglycerate (0.017 *M*). Air, 38°. Ad=adenylic acid. Coz=cozymase. Pgl=phosphoglyceric acid. Ppyr=phosphopyruvic acid.

Time min.	Ad added (mol. $\times 10^{-3}$)	Coz added (mol. $\times 10^{-3}$)	Inorganic P mg.	Pgl P mg.	Ppyr P mg.	Increase of inorganic P mg.
0	—	—	0.37	0.74	0.00	—
30	—	—	0.40	0.51	0.20	0.03
30	0.14	—	1.16	0.00	0.00	0.79
30	—	0.28	1.18	0.00	0.00	0.81

formation of iodine-labile P (phosphopyruvate-P) in the absence of "adenine nucleotide", and the obvious necessity of this (in catalytic concentrations) for the reaction causing liberation of inorganic phosphate. The occurrence of this reaction and the high rate at which it takes place³ indicates that the brain dispersions contain a strongly active adenosinetriphosphatase. This makes it impossible to decide whether oxidation of pyruvate in brain dispersions is accompanied by phosphorylation of adenylic acid to adenosinepolyphosphate. When dialysed brain dispersions are incubated with adenylic acid containing

Table X. *Transfer of phosphate from phosphoglyceric acid to adenylic acid in dialysed extract from pigeon's brain*

1 ml. enzyme (dialysed 3 hr.) to 1.5 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.0066 *M*), phosphoglycerate (0.017 *M*; 0.8 mg. P), and adenylic acid (0.0028 *M*; 0.35 mg. P). Air, 30°.

Time min.	Inorganic P mg.	Phosphopyruvic P mg.	Pyrophosphate P mg.
0	0.35	0.00	0.00
15	0.44	0.03	0.39

0.17 mg. P, at 30° for 5 min., some easily hydrolysable P (7 min., 100°, *N* HCl) accumulates in the presence, but not in the absence, of pyruvate; the quantities

¹ Detected qualitatively by the nitroprusside reaction.

² With preparations of *Bacterium Delbruckii*, which oxidize pyruvic acid to acetic acid and CO_2 , Lipmann [1937] found that phosphopyruvic acid was not oxidized.

³ Liberation of PO_4 was found to be complete in 10 min. in other experiments.

formed are, however, very small, generally amounting to only about 0.01 mg. P. On the other hand, the adenosinetriphosphatase is insoluble¹ and, when the dispersions are centrifuged at high speed (15,000 rev./min.) for a short time, the clear solution is no longer able to set free inorganic phosphate from phosphoglyceric acid significantly; but as the enzymes catalysing the conversion of phosphoglyceric into phosphopyruvic acid and transferring phosphate from the latter to adenylic acid are present in it, adenylic acid is readily phosphorylated (Table X).

With the demonstration of the formation of phosphopyruvic from phosphoglyceric acid, and the transfer of phosphate from phosphoglyceric to adenylic acid in brain preparations, which completes the evidence supplied by the experiments of Mazza & Valeri [1935], Euler *et al.* [1936] and Meyerhof [1938], we now have the final proof for the existence in brain of a glycolytic enzyme system which, at least qualitatively, is identical with that of skeletal muscle. Evidence in favour of a similar glycolytic mechanism in the retina has been recently advanced by Süllmann & Vos [1939].

As shown in Table IX, cozymase is as active as adenylic acid in catalysing the liberation of inorganic phosphate from phosphoglyceric acid in brain dispersions. This is not the case with muscle extracts [Schlenk *et al.* 1937], hence it seems likely that brain dispersions split cozymase to some extent with liberation of cophosphorylase.

2. EXPERIMENTS ON MAMMALIAN BRAIN AND KIDNEY (with technical assistance by R. W. Wakelin)

Substantially the same results as with pigeon's brain dispersions, have been obtained with dispersions prepared from rabbit's brain. The oxidation of pyruvate, after dialysis, requires the addition of phosphate, fumarate and adenine nucleotide as shown in Table XI. The difference from pigeon's brain dispersions is that, whereas, after periods of dialysis not exceeding 1.5 hr., cozymase is practically as active as adenylic acid, and can replace it molecule for molecule (Table XI, Exp. 2a), its activity is enormously reduced after dialysis periods of over 4 hr. (Table XI, Exps. 2b, 3b). Thus it seems that, in rabbit's brain dispersions, the enzyme splitting off cophosphorylase from cozymase is easily inactivated by dialysis; in general the rabbit preparations seem less stable after dialysis.

The effect of fumarate and adenylic acid has also been obtained in dispersions from guinea-pig's brain, even without dialysis² (Table XII), but we have failed to obtain active dispersions from rat's brain even with several modifications. Although the O₂ uptake of both brei and slices of rat brain is markedly increased by pyruvate, this is only so to a very small extent with dispersions. The explanation for this must probably be sought in the much higher instability of some enzymic component of the system.

In preliminary experiments with dispersions of rabbit kidney cortex, the necessity of fumarate and adenylic acid in the pyruvate oxidation system has also been demonstrated. The effect of adenylic acid is shown in Fig. 4. Table XIII shows the large increase in the net oxidation of pyruvate obtained when both

¹ The same finding, as regards the adenosinetriphosphatase of muscle, has been recently reported by Engelhardt & Ljubimowa [1939].

² In two experiments out of three.

fumarate and adenylic acid are added. The kidney dispersions were prepared in exactly the same way as those of brain, except that 1 part tissue was ground with only 2 volumes of 0.9 % KCl.

Table XI. *Effect of phosphate, fumarate, adenylic acid and cozymase on the oxidation of pyruvate by dialysed dispersions from rabbit's brain*

1.5 ml. enzyme* (dialysed for various periods) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$). Air, 38°. (5 out of 10 experiments quoted.)

Exp.	Duration of dialysis hr.	(mol. $\times 10^{-3}$)					μ l. O_2 uptake in 20 min.
		Pyruvate	Phosphate	Fumarate	Adenylic acid	Cozymase	
1	3.75	—	50	5	0.14	—	27
		9.1	—	—	0.14	—	37
		9.1	50	—	0.14	—	52
		9.1	—	5	0.14	—	54
		9.1	50	5	0.14	—	153
2a†	1.5	—	50	5	0.14	0.14	59
		9.1	50	5	—	—	58
		9.1	50	5	0.14	—	198
		9.1	50	5	—	0.14	153
		9.1	50	5	0.14	0.14	224
2b	4.5	—	50	5	0.14	0.14	30
		9.1	50	5	—	—	46
		9.1	50	5	0.14	—	163
		9.1	50	5	—	0.14	62
		9.1	50	5	0.14	0.14	204
3a‡	1.25	—	50	5	1.4	0.14	59
		9.1	50	5	—	—	48
		9.1	50	5	1.4	—	254
		9.1	50	5	—	0.14	140
		9.1	50	5	1.4	0.14	244
3b‡	4.5	—	50	5	1.4	0.14	42
		9.1	50	5	—	—	53
		9.1	50	5	1.4	—	175
		9.1	50	5	—	0.14	65
		9.1	50	5	1.4	0.14	185
4§	4	—	50	5	0.14	—	30
		9.1	50	—	—	—	35
		9.1	50	5	—	—	46
		9.1	50	—	0.14	—	41
		9.1	50	5	0.14	—	99
5a	0.7	—	50	5	0.7	0.14	94
		9.1	50	5	—	—	62
		9.1	50	5	0.7	—	271
		9.1	50	5	—	0.14	183
		9.1	50	5	0.7	0.14	270
5b	1.7	—	50	5	0.7	0.14	63
		9.1	50	5	—	—	64
		9.1	50	5	0.7	—	249
		9.1	50	5	—	0.14	164
		9.1	50	5	0.7	0.14	248

* The amount of brain tissue equivalent to 1.5 ml. enzyme varied in the different experiments (from 190 to 316 mg.) but the O_2 uptake has been referred throughout to 300 mg. tissue for better comparison with the experiments on pigeon's brain.

† 2a and 2b, 3a and 3b, and 5a and 5b represent respectively samples from the same brain. In another experiment the cophosphorylase effect of cozymase had practically disappeared after 1.75 hr. dialysis.

‡ Dialysis against 0.9 % KCl.

§ Malate used instead of fumarate.

Table XII. *Effect of fumarate and adenylic acid on the oxidation of pyruvate by undialysed dispersions from guinea-pig's brain*1.5 ml. enzyme to 2 ml. with additions including phosphate (0.05 *M*). Air, 38°.

Pyruvate (mol. $\times 10^{-3}$)	Fumarate (mol. $\times 10^{-3}$)	Adenylic acid (mol. $\times 10^{-3}$)	μ l. O ₂ uptake in 20 min.
—	0.5	0.14	71
9.1	—	—	61
9.1	0.5	—	82
9.1	0.5	0.14	197

Fig. 4. Effect of adenylic acid on the oxidation of pyruvate by dialysed dispersions from kidney cortex (rabbit). 1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions. The complete system (curve I) contains enzyme, 0.1 mg. Mg⁺⁺ (as MgCl₂), phosphate (0.05 *M*), fumarate (0.005 *M*), adenylic acid (0.0028 *M*) and pyruvate (0.0091 *M*). Curve II: no adenylic acid. Curve III: no pyruvate. Air, 38°.Table XIII. *Effect of fumarate and adenylic acid on the oxidation of pyruvate by dialysed dispersions from kidney cortex (rabbit)*1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions including 0.1 mg. Mg⁺⁺ (as MgCl₂), and phosphate (0.05 *M*). Air, 38°.

Sample	μ l. O ₂ uptake in 20 min.	
	No addition	Fumarate (0.005 <i>M</i>) + Adenylic acid (0.0028 <i>M</i>)
Enzyme	42	160
Enzyme + pyruvate (0.0091 <i>M</i>)	172	472
Net O ₂ uptake of pyruvate	130	312

Increase of O₂ uptake of pyruvate in presence of fumarate + adenylic acid 182 μ l. (+ 140 %).

DISCUSSION

The role of inorganic phosphate and "adenine nucleotide" in pyruvate oxidation. The necessity of both inorganic phosphate and adenine nucleotide for the oxidation of pyruvate in animal tissues, which is here shown for the first time, leads to the hitherto unsuspected assumption that phosphorylation reactions must play a part in oxidative breakdown of carbohydrate, beyond the stage of

phosphopyruvic acid. As regards bacteria Lipmann [1937] had shown that inorganic phosphate is required for the oxidative decarboxylation of pyruvate to acetic acid and CO_2 by washed acetone preparations of *Bacterium Delbrückii*; the possibility that a phosphorylation to phosphopyruvic acid, prior to the dehydrogenation, might be necessary was excluded by his finding that phosphopyruvic acid itself was not oxidized by the bacterial preparations. Lipmann [1938] observed later that decarboxylation of pyruvic acid by yeast does not require inorganic phosphate. More recently [Lipmann, 1939, 1] he was able to show that, if added to the (adenosinetriphosphatase-free) bacterial preparations, in the presence of pyruvate, adenylic acid is phosphorylated to adenosinepolyphosphate.

Lipmann's observations, however, differ from our own in one important respect. In the absence of adenylic acid, the dehydrogenation of pyruvate by the bacterial enzymes was not lower than in its presence, as one would expect if there existed a true coupling between pyruvate dehydrogenation and the phosphorylation of adenylic acid [cf. for instance, Meyerhof, 1937]. In our experiments with brain dispersions, on the other hand, the oxidative removal of pyruvate is very small in the absence of adenylic acid and is greatly increased when the nucleotide is added. Furthermore, the net O_2 uptake of pyruvate is increased to an even greater extent, so that pyruvate is being more completely oxidized. Thus, whereas "adenine nucleotide" may be necessary for the oxidative decarboxylation of pyruvate in animal tissues, it also seems to be required in further oxidative stages or other reactions involved in the oxidation.

The possibility that, in muscle respiration, phosphorylation may be coupled with reactions other than the dehydrogenation of triosephosphate [cf. Lénnerstrand, 1937; Needham & Lu, 1938], is suggested by experiments of Belitzer [1939], who found that addition of creatine to muscle brei suspensions in phosphate led to both increased O_2 uptake and synthesis of phosphocreatine. This happened with lactate as substrate, although to a less extent, even in the presence of 0.00025 *M* bromoacetate which completely inhibited glycolysis. Whether such low concentrations of bromoacetate would also inhibit aerobic oxidation of triosephosphate is not stated.

If pyruvate oxidation is coupled with phosphorylation of "adenine nucleotide", then the energy of the main reaction of respiration would also seem to be made available through the adenylic acid and creatine phosphate systems.

Quite recently Lipmann [1939, 2] has suggested acetylphosphate as an intermediate in the dehydrogenation of pyruvate by *B. Delbrückii* preparations and, possibly, by animal tissues, but Ochoa *et al.* [1939] could not find evidence to support the latter view. The evidence at present available favours the possibility of an intermediary phosphorylation in the oxidation of pyruvate by both bacteria and animal tissues and, in spite of differences in the experimental results, it would seem that a common phosphorylated intermediate, which does not appear to be acetylphosphate, might be formed in both cases [Ochoa *et al.* 1939]. Recent experiments by one of us¹ show that, in brain dispersions poisoned with NaF, and in presence of pyruvate and the other components of the oxidation system, there is some disappearance of inorganic phosphate with equivalent formation of an easily hydrolysable ester, which is neither alkali- nor iodine-labile. The absolute amounts of ester formed are small but the ratio mol. pyruvate disappeared/atoms P esterified is only about 3. Further experiments in this direction are in progress.

¹ Ochoa, unpublished results.

The role of C₄ dicarboxylic acids. Whether the system of C₄ dicarboxylic acids is only acting in a first stage of pyruvate dehydrogenation or whether (as is the case with "adenine nucleotide") it is also involved in further stages, has not been accurately investigated, but we have some evidence that the latter is the case.

On the other hand, it appears that C₄ dicarboxylic acids are acting, in brain pyruvate oxidation, in the way suggested by Szent-Györgyi and his collaborators [cf. Szent-Györgyi, 1937], i.e. in a reversible catalytic system of hydrogen carriers, rather than as intermediates in a catalytic cycle such as the citric acid cycle of Krebs & Johnson [1937]. If the latter occurred one would expect citric acid (also α -ketoglutaric acid) to be at least as active as any of the C₄ dicarboxylic acids. This is not the case in spite of the fact that brain contains both aconitase [Johnson, 1939] and isocitric dehydrogenase [Adler *et al.* 1939].

SUMMARY

1. Dialysis of pigeon brain dispersions for short periods greatly reduces their power to oxidize pyruvate. Full reactivation can, however, be obtained by addition of inorganic phosphate + a C₄ dicarboxylic acid (succinate, fumarate, malate) + "adenine nucleotide" (adenylic acid or adenosinetriphosphate). The same is true for dispersions of mammalian brain (rabbit).

2. In the presence of the other components of the system, adenine nucleotide not only increases the oxidative removal of pyruvate considerably, but makes its oxidation more complete. Thus, whereas in the absence of adenine nucleotide the ratio mol. O₂ taken up/mol. pyruvate removed is 0.75, in its presence, the ratio is increased to 1.5 (at 28°).

3. The ratio mol. O₂/mol. pyruvate of the excess metabolism produced by adenylic acid is 2.1, i.e. near the theoretical value of 2.5 for complete oxidation of pyruvate to CO₂ + H₂O.

4. Cozymase can replace adenylic acid and (in pigeon's brain dispersions) the former is, molecule for molecule, even more active than the latter. This effect of cozymase is probably due to enzymic splitting of cophosphorylase ("adenine nucleotide"). In rabbit's brain dispersions, cozymase replaces adenylic acid only after periods of dialysis not exceeding 1.5 hr. After 4.5 hr. dialysis this is no longer the case.

5. The possibility that cozymase, acting as codehydrogenase, may be required at some stage of pyruvate oxidation is discussed.

6. Phosphoglycerate is oxidized by pigeon brain dispersions almost as readily as is pyruvate. This is due to its rapid conversion into pyruvate + H₃PO₄. The formation of phosphopyruvic from phosphoglyceric acid, in brain dispersions, and the transfer of phosphate from phosphoglyceric to adenylic acid, in brain extracts, have been shown to occur in the same way as in skeletal muscle.

7. Citrate or α -ketoglutarate are much less active than any of the C₄ dicarboxylic acids in catalysing the oxidation of pyruvate by brain dispersions. This is discussed.

8. The necessity of fumarate and adenylic acid for the oxidation of pyruvate by kidney cortex dispersions (rabbit) has been demonstrated in preliminary experiments.

One of us (S. O.) wishes to thank the Nuffield Trustees for a personal grant. We are indebted to the Nuffield Trustees, the Rockefeller Foundation, and the Medical Research Council for grants in aid of this work and to Mr H. W. Kinnersley, Mr R. W. Wakelin and Mr Clarke for help with the pigeons.

REFERENCES

- Adler, Euler, Günther & Plass (1939). *Biochem. J.* **33**, 1028.
Annau & Erdös (1939). *Hoppe-Seyl. Z.* **257**, 111.
Banga, Ochoa & Peters (1939, 1). *Biochem. J.* **33**, 1109.
——— (1939, 2). *Nature, Lond.*, **144**, 74.
Belitzer (1939). *Enzymologia*, **6**, 1.
Engelhardt & Ljubimowa (1939). *Nature, Lond.*, **144**, 668.
Euler, Günther & Vestin (1936). *Hoppe-Seyl. Z.* **240**, 265.
Johnson (1939). *Biochem. J.* **33**, 1046.
Jowett & Quastel (1937). *Biochem. J.* **31**, 275.
Krebs & Johnson (1937). *Enzymologia*, **4**, 148.
Lennerstrand (1937). *Naturwissenschaften*, **25**, 347.
Lipmann (1937). *Enzymologia*, **4**, 65.
——— (1938). XVIth Internat. Physiol. Congress. *Kongressbericht II*, 161.
——— (1939, 1). *Nature, Lond.*, **143**, 281.
——— (1939, 2). *Nature, Lond.*, **144**, 381.
Lohmann & Jendrassik (1926). *Biochem. Z.* **178**, 419.
Long (1938). *Biochem. J.* **32**, 1711.
——— Ochoa & Peters (1939). *J. Physiol.* **96**, 7 P.
Mazza & Valeri (1935). *Arch. Sci. biol. (Napoli)*, **21**, 443.
McGowan (1937). *Biochem. J.* **31**, 1627.
——— & Peters (1937). *Biochem. J.* **31**, 1637.
Meyerhof (1937). *Naturwissenschaften*, **25**, 443.
——— (1938). *Bull. Soc. Chim. biol.* **20**, 1335.
——— & Ohlmeyer (1936). *Naturwissenschaften*, **24**, 741.
——— (1937). *Biochem. Z.* **290**, 334.
Needham & Lu (1938). *Biochem. J.* **32**, 2040.
Ochoa (1939). *Nature, Lond.*, **144**, 834.
——— Peters & Stocken (1939). *Nature, Lond.*, **144**, 750.
Ohlmeyer & Ochoa (1937). *Biochem. Z.* **293**, 338.
Peters, Rydin & Thompson (1935). *Biochem. J.* **29**, 63.
Schlenk, von Euler, Heiwinkel, Glein & Nyström (1937). *Hoppe-Seyl. Z.* **247**, 23.
Straub (1936). *Hoppe-Seyl. Z.* **244**, 117.
Süllmann & Vos (1939). *Enzymologia*, **6**, 246.
Szent-Györgyi (1937). Studies on biological oxidations.
Vercellone & Neuberg (1935). *Biochem. Z.* **280**, 161.