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Pyruvate Oxidation in Ox Heart

BY Q. H. GIBSON AND C. LONG, *Department of Biochemistry, The Queen's University, Belfast*

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During the past decade, the *in vitro* oxidation of pyruvic acid by molecular O_2 in the presence of surviving animal tissues has been extensively studied. Published work indicates that, in general, the requirements of these pyruvate oxidation systems for maximal O_2 uptake are (i) inorganic phosphate, (ii) adenine nucleotide, (iii) a C_4 -dicarboxylic acid and possibly (iv) magnesium ions (Banga, Ochoa & Peters, 1939; Smyth, 1940; Ochoa, 1943; Long, 1943, 1946).

Although it is now established that a trace of a C_4 -dicarboxylic acid is needed as a catalyst for maximal O_2 uptake in the pyruvate oxidation system, the effect of the C_4 -acids on the rate of pyruvate disappearance is not yet settled. Thus Annau, Banga, Blazsó, Bruckner, Laki, Straub & Szent-Györgyi (1936), working with rabbit liver, consistently found no increase in the rate of pyruvate utilization when fumarate was added, and Long (1946) obtained the same result with dialyzed pigeon brain suspensions. On the other hand, Krebs & Eggleston (1940), using pigeon breast muscle, reported an increased rate of pyruvate disappearance in one experiment and a decreased rate of disappearance in another.

The present paper summarizes the results of an investigation of the behaviour of a dialyzed ox heart preparation respiring in a medium containing pyruvate. It has been found that maximal activity requires the addition of inorganic phosphate, adenine nucleotide, fumarate and magnesium ions, and that the increased rate of O_2 consumption produced by fumarate addition is accompanied by a corresponding increase in the rate of pyruvate disappearance.

EXPERIMENTAL

Materials

Na pyruvate, Na fumarate and $MgCl_2$ were pure analytical specimens. Ba adenosine triphosphate was prepared from rabbit skeletal muscle by the method of DuBois, Albaum & Potter (1943). When required, the barium was removed as $BaSO_4$ and a stock solution prepared at a concentration of 0.01 M.

Methods

Ox heart was placed on ice as soon as possible after the death of the animal, and on arrival at the laboratory was rapidly washed and freed from fat and connective tissue. The muscle was minced finely in a Latapie mincer, the cutting blade of which was driven by an electric motor. (It has been found that the degree of subdivision of the tissue is a matter of considerable importance: thus if the mincing process is carried too far, the respiration rate of the resulting preparation is low, while if it is insufficient subsequent dialysis will fail to remove substrates and activators.)

A portion of the mince (2.8 g.) was suspended in 25.2 ml. ice-cold 0.9% KCl containing 0.04% $MgCl_2 \cdot 6H_2O$ (tissue diluted 1 in 10), and several 5 ml. portions of the suspension dialyzed in collodion sacs against 4 l. of 0.4% KCl containing 0.002 M- $MgCl_2$ for 3 hr. at 2°. The dialyzer was gently rocked throughout this period so that the movement of an air bubble from one end of the sac to the other kept the tissue suspension in motion.

Portions of the dialyzed suspension, usually 2 ml., were pipetted into the manometric flasks containing substrates, activators, etc. (total volume, 3.4 ml.) and 0.2 ml. 2 N-KOH with filter paper slip was placed in the centre well. Respiration was conducted at 28°, using Barcroft manometers. After 15 min. equilibration, respiration was allowed to continue for a further 2 hr. Pyruvate was estimated by the

method of Long (1942). Respiratory quotients were determined by the direct method (Dixon, 1943) and also by the method of Dickens & Šimer (1931).

RESULTS

The effect of inorganic phosphate concentration

A typical curve is shown in Fig. 1, where the net O_2 uptake ($\mu\text{l./g. tissue/90 min.}$) is given for various concentrations of inorganic phosphate (pH 7.4) in the medium. The maximal rate of respiration is

Fig. 1. The effect of inorganic phosphate concentration on the oxygen uptake by dialyzed ox heart suspension. The oxygen uptakes are net figures for 2 ml. suspension respiring in air at 28° in $\mu\text{l./g. wet weight}$ for 90 min. The phosphate concentrations are expressed in molarity $\times 10^{-4}$. Each manometer contained 0.000375M-adenosine triphosphate, 0.004M-Na pyruvate, 0.0012M-MgCl₂ and 0.0005M-Na fumarate.

obtained at a phosphate concentration of 0.01–0.05M. The shape of the curve indicates that the O_2 uptake would be very small, and probably zero, in the complete absence of inorganic phosphate. The residual phosphate at the end of an experiment was usually of the order of $5 \times 10^{-4}\text{M}$, and the effects of lower concentrations could not be examined. When the O_2 consumption at different phosphate concentrations is plotted against duration of respiration, it appears that the effect of inorganic phosphate is more pronounced in the later part of the experiment, i.e. that it exerts a stabilizing influence on the respiration of the tissue suspension.

It should be noted, however, that in these experiments the respiration medium was unbuffered before the addition of the inorganic phosphate, pH 7.4 being outside the range of glycine (pH 8.24–10.14) and borate (pH 7.62–9.24) buffers. As the phosphate was added in the form of a buffer of pH 7.4 it is possible that the effect produced is in part due to the stabilization of pH. This would explain the observation that the most marked effect

Fig. 2. The effect of adenosine triphosphate on the respiration of ox heart suspension. The oxygen uptakes are gross figures in $\mu\text{l./g. tissue}$ at the concentrations of added adenosine triphosphate shown. Conditions as in Fig. 1, except that each manometer contained 0.05M-phosphate buffer pH 7.4.

of phosphate addition was found in the later stages of the experiment, since lactic acid might be formed from residual carbohydrate during incubation.

The effect of magnesium ions

In the early part of the present work, much trouble was experienced in obtaining dialyzed suspensions of heart muscle showing a stable respiration, and many preparations were only slightly active. It was found that inactivation took place during dialysis against 0.4% KCl: storage at the

temperature of dialysis did not inactivate the suspension. The addition of Mg^{++} to the dialysis fluid was found to prevent this loss of activity. The effect produced is shown by the increase in gross O_2 uptake from 275 μ l./g. tissue/hr. (av. of 12 experiments) in

In every case an increased rate of disappearance of pyruvate has been observed following the addition of fumarate (Table 1, columns 5 and 10). In this respect, ox heart differs markedly from pigeon brain (Long, 1946).

Table 1. Net molecular O_2 /pyruvate ratios in the presence and absence of fumarate

Each flask contained 2 ml. ox heart suspension (200 mg. wet weight of mince), 0.000375M-adenosine triphosphate, 0.05M-phosphate buffer, pH 7.4 and 0.0012M- $MgCl_2$. Final concentration of added fumarate, 0.0005M; pyruvate, 0.004M. Total volume 3.4 ml. Respiration in air at 28°, for 2 hr.

Exp.	Fumarate absent					Fumarate present				
	O_2 uptake (μ mol.)			Pyruvate disappearance (μ mol.)	Net O_2 /pyruvate ratio	O_2 uptake (μ mol.)			Pyruvate disappearance (μ mol.)	Net O_2 /pyruvate ratio
	Res.	Pyr.	Net			Res.	Pyr.	Net		
1	3.1	10.4	7.3	4.1	1.78	3.9	15.5	11.6	6.3	1.85
2	—	—	—	—	—	2.0	12.0	10.0	5.8	1.72
3	4.2	6.6	2.4	3.9	0.62	10.0	20.9	10.9	8.8	1.24
4	—	—	—	—	—	5.6	15.5	9.9	7.0	1.41
5	1.6	3.1	1.5	1.0	1.50	1.9	15.4	13.5	6.1	2.22
6	5.1	7.3	2.2	3.7	0.60	8.3	19.6	11.3	7.9	1.43
7	4.8	11.4	6.6	5.2	1.27	9.1	26.4	17.3	10.3	1.68
8	2.5	2.8	0.3	2.7	0.11	6.7	12.2	5.5	4.9	1.12
9	—	—	—	—	—	3.0	14.1	11.1	6.0	1.85
10	3.4	7.5	4.1	2.7	1.52	4.8	18.0	13.2	6.7	1.97
11	—	—	—	(Undialyzed)	—	16.4	16.6	0.2	6.1	0.03
12	—	—	—	(Undialyzed)	—	19.6	22.3	2.7	8.5	0.32

the absence of magnesium during dialysis, to 1220 μ l./g./hr. (av. of 17 experiments) in the presence of magnesium. It is concluded, therefore, that in the absence of Mg^{++} the enzyme system is irreversibly inactivated to a marked degree. The necessity for the presence of magnesium during the oxidation of pyruvate could not, of course, be demonstrated except in experiments where dialysis had been conducted against a magnesium-free fluid: under these conditions, the addition of Mg^{++} appeared to produce some stimulation of respiration, but owing to the virtual inactivity of the preparations the results were not reproducible.

The effect of adenosine triphosphate concentration

The addition of small amounts of adenosine triphosphate to the enzyme system is accompanied by a marked stimulation of the rate of respiration. The effect of increasing concentrations of this activator on the rate of O_2 consumption is shown in Fig. 2.

The effect of fumarate concentration

Catalytic amounts of fumarate (5×10^{-4} M), when added to the ox heart enzyme system respiring in pyruvate, cause a marked increase in the rate of O_2 consumption. The effect of different concentrations of Na fumarate on the rate of respiration is shown in Fig. 3. It will be seen that almost maximal activity is obtained with exceedingly low fumarate concentrations. The rate of pyruvate utilization has also been studied in the presence and absence of fumarate.

These results show that satisfactory rates of O_2 uptake and pyruvate utilization may be obtained with the following medium (final concentration in brackets): KCl (0.03M), Na_2HPO_4 - KH_2PO_4 buffer, pH 7.4 (0.05M with respect to phosphate), $MgCl_2$ (0.0012M), adenosine triphosphate (0.000375M), Na fumarate (0.0005M), and Na pyruvate (0.02M). Under these conditions, the rate of O_2 uptake was about 1.2 ml./g. moist tissue/hr., corresponding to $Q_{O_2}^{28^\circ} = \text{approx. } -7$. Having regard to the difference in temperature, the activity is of the same order as that obtained by Smyth (1940) for undialyzed sheep heart suspensions respiring at 38°.

The O_2 /pyruvate ratio

Simultaneous determinations of the amount of O_2 absorbed and the quantity of pyruvate disappearing were made both in the presence and absence of fumarate, and the molecular O_2 /pyruvate ratio calculated. Using the normal procedure for obtaining the net O_2 uptake by subtracting the residual O_2 uptake from the total O_2 uptake in the presence of pyruvate, very divergent values for the net O_2 /pyruvate ratio were found in different experiments (Table 1). It was observed, however, that the ratio varied with the extent of the residual O_2 uptake, being small when the residual uptake was large and *vice versa*. The net ratio is given by

$$\frac{O_2 \text{ (total)} - O_2 \text{ (residual)}}{\text{pyruvate disappearance}}$$

This net ratio is the true ratio provided the residual O_2 uptake is not inhibited by pyruvate. If the residual O_2 uptake is at all inhibited in the presence of pyruvate, kO_2 (residual) rather than O_2 (residual) should be subtracted from the total O_2 uptake in the

Fig. 3. The effect of fumarate on the oxygen uptake of ox heart suspension. The net oxygen uptakes are given in $\mu\text{l./g.}$ tissue for the concentrations of added fumarate shown. Other conditions as for Fig. 1, except that each manometer contained 0.05M-phosphate buffer pH 7.4.

above expression, where k is the fraction of the residual uptake not inhibited by pyruvate. If O_2 (residual) is subtracted instead of kO_2 (residual), the net ratio will always be too small, and the larger the O_2 (residual) as a fraction of O_2 (total), the smaller the net O_2 /pyruvate ratio will become. This corresponds to the present case and suggests that the residual O_2 uptake is in fact inhibited in the presence of pyruvate. It was found further that a reasonably linear relationship existed between the net O_2 /pyruvate ratio and the size of the residual O_2 uptake, the latter being expressed as a fraction of the total O_2 uptake in the presence of pyruvate (Fig. 4). Extrapolation of the curve to the point of intersection with the ordinate would correspond to the molecular ratio expected with a preparation which had been dialyzed so completely that the residual O_2 uptake was zero. A line fitted to the data by the method of least squares intersects the ordinate at a value of 2.505. This figure for the net ratio corresponds with the value for complete oxidation of pyruvate according to the equation

Another inference which may be drawn from the graph is that the O_2 /pyruvate ratio is not dependent on the presence or absence of fumarate, since all the points lie on the same line. This finding is in contrast to the results obtained for pigeon brain suspensions (Long, 1943, 1946), where the oxidation of pyruvate was found to be less complete in the absence of fumarate. In addition, further analysis of the graph shows that the inhibition of the residual O_2 uptake may be obtained from the slope and position of the curve. In our case, we deduce that the residual O_2 uptake is completely inhibited in the presence of pyruvate.

Fig. 4. The molecular ratio O_2 /pyruvate. The net value for the molecular ratio O_2 /pyruvate is shown plotted against the ratio O_2 residual/ O_2 total. ● fumarate (0.0005M) present; ○ fumarate absent; ▲ undialyzed heart suspension.

The respiratory quotient

The R.Q. for pyruvate oxidation in the presence of ox heart suspensions was determined by the direct method and also by the method of Dickens & Šimer (1931). The results are shown in Table 2. Since we consider that the residual respiration is completely inhibited in the presence of pyruvate, net figures have no significance, and the R.Q. in the presence of pyruvate is the only value of importance. From

Table 2. R.Q. for pyruvate oxidation

The R.Q. figures were obtained either by the method of Dickens & Šimer (1), or by the direct method (2). For the method of Dickens & Šimer, 1 ml. tissue suspension was used; in the direct method, 2 ml. were taken. The uptakes are in μ l. O_2 and CO_2 /2 hr. at 28° . Where both methods were used, the figures refer to 2 ml. suspension, the average value being given. Other conditions are as in Table 1. 0.0005M-fumarate was present throughout.

Residual			Pyruvate present			
O_2 uptake (μ l.)	CO_2 output (μ l.)	R.Q.	O_2 uptake (μ l.)	CO_2 output (μ l.)	R.Q.	Method
76.8	74.7	0.97	255.0	266.0	1.04	(1)
22.8	19.9	0.87	97.3	114.6	1.18	(1)
165.0	139.0	0.84	697.0	789.0	1.13	(1), (2)
88.2	89.0	1.01	221.5	267.0	1.21	(1)
24.6	20.6	0.84	193.0	219.5	1.14	(1)
77.9	76.3	0.98	381.6	457.0	1.20	(1), (2)
64.0	48.7	0.76	412.0	483.0	1.17	(1), (2)
Average 0.88			Average 1.15			

Table 2, the R.Q. is seen to be of the order 1.2. This also agrees with the calculated value for complete oxidation.

DISCUSSION

The results quoted in the experimental part of this paper lend themselves to a comparison between the pyruvate oxidation processes in ox heart and in other tissues. The tissue which has been studied most intensively under comparable conditions is finely divided pigeon brain suspension, which after dialysis requires inorganic phosphate, adenine nucleotide and a C_4 -dicarboxylic acid for maximal rate of respiration and pyruvate utilization (Banga *et al.* 1939; Long, 1943, 1946).

There would appear to be some difference between these two tissues in their requirement for magnesium, since pigeon brain is not inactivated by dialysis in the absence of Mg^{++} . The addition of Mg^{++} after dialysis has not been found to affect the respiration of pigeon brain suspensions (Long, unpublished), although Ochoa (1939) has claimed that Mg^{++} is necessary in experiments carried out under similar conditions. It would seem therefore, either that Mg^{++} is not necessary for pigeon brain, or that it is more firmly bound to the enzyme than in heart.

Heart and brain preparations also differ in the effects produced by the C_4 -dicarboxylic acids on the course of pyruvate oxidation in these tissues. Most animal tissues, respiring in a medium containing pyruvate, respond to the addition of fumarate, the O_2 uptake being generally at least doubled. Heart and brain behave similarly in this respect. They differ, however, in the effect produced by fumarate on the rate of pyruvate utilization. Thus in brain tissue, the extent of pyruvate disappearance is not increased by fumarate addition, the effect of which is to bring about complete rather than partial oxidation of that amount of pyruvate which is utilized in the absence of fumarate (Long, 1946). In heart, on the other hand, fumarate addition

increases the actual rate of pyruvate disappearance. It does not affect the completeness of the pyruvate oxidation, since even without added fumarate complete oxidation takes place. It would appear, therefore, that animal tissues differ in this respect, heart and possibly pigeon breast muscle (Krebs & Eggleston, 1940) contrasting with brain (Long, 1946) and liver (Annau *et al.* 1936).

The method adopted in the present work for determining the true O_2 /pyruvate ratio is of some general interest, since it may be applied to the study of any tissue in which the residual O_2 uptake of different preparations varies over a fairly wide range. It is clear that if in our experiments the normal method of subtracting the residual from the total O_2 uptake had been followed, it would have been wrongly concluded that pyruvate was incompletely oxidized by all our preparations, and that the addition of fumarate led to a relatively more complete oxidation of pyruvate. The residual respiration of heart muscle may be due to the presence of reserve carbohydrate in a non-dialyzable form which is broken down during the respiration period and oxidized as pyruvate. The addition of pyruvate in large amounts would almost certainly inhibit the formation of pyruvate from residual carbohydrate, and simple addition of the residual O_2 consumption to that due to pyruvate would not be expected to occur. It would seem that in work of this kind it is advisable to examine the data carefully before using net values calculated by subtracting the residual from the total O_2 uptake. A reinvestigation of the results for pigeon brain (Long, 1943, 1946) has indicated that the normal procedure of subtracting the residual O_2 uptake was a valid procedure in that particular case, since the same values for the O_2 /pyruvate ratio were obtained when the method used for the heart preparations was employed. It should be pointed out, however, that the latter method is difficult to apply in the case of brain because of the relative constancy of the residual O_2 uptake. The

method used in calculating the results for the heart preparations, though offering uncertainties of its own, is probably nevertheless the more general and desirable procedure.

SUMMARY

1. The greatest activity of the pyruvate oxidation system by finely divided dialyzed ox heart suspensions is observed in the presence of inorganic phosphate, adenine nucleotide and fumarate added simultaneously.

2. The presence of Mg^{++} is necessary to prevent irreversible inactivation of the enzyme during dialysis.

3. Addition of catalytic amounts of fumarate (0.0005M) greatly increases both the O_2 uptake and pyruvate disappearance.

4. The pyruvate is completely oxidized to CO_2 and water, the molecular ratio O_2 /pyruvate deduced by extrapolation from the experimental data being 2.5 and the R.Q. 1.2.

5. A comparison is made between the course of pyruvate oxidation in ox heart and pigeon brain suspensions.

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Haemolysis and Fixation of Erythrocytes by X-Rays

By L. HALBERSTAEDTER AND J. LEIBOWITZ, *Department of Radiology, The Hebrew University, Jerusalem*

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It has previously been reported (Halberstaedter & Goldhaber, 1943; Halberstaedter, 1944) that following irradiation with an X-ray dose of 2×10^6 r. at high intensity, erythrocytes *in vitro* undergo either haemolysis or fixation. The type of the response depends on the dose of X-rays, on the concentration of the erythrocyte suspension and on the medium in which they are suspended during irradiation. At high cell concentrations ($> 0.5\%$) the effect of the irradiation is always haemolysis independently of the nature of the medium. At low concentrations ($< 0.5\%$), on the other hand, the type of the response obtained depends on the medium in which the cells are suspended. At low concentration, the erythrocytes are resistant to haemolysis if irradiated in a medium of 0.9% sodium chloride solution, but undergo complete haemolysis if irradiated in a medium of isotonic glucose or 1 vol. serum : 3 vol. of 0.9% sodium chloride solution.

Continuing this investigation, the influence of different media on the response of erythrocytes to X-ray irradiation has been studied. Experiments

were made to determine what serum constituent is responsible for the haemolytic reaction of erythrocytes which are irradiated at low concentration with an X-ray dose of 2×10^6 r.

EXPERIMENTAL

Blood. Fresh rabbit or human blood was defibrinated, the erythrocytes washed twice with 0.9% NaCl and suspended in concentrations of 0.1% and 1% (v/v) in the different media. Small hanging drops under mica slips were irradiated and the effects observed with the microscope.

X-rays. Irradiation was carried out by means of a demountable X-ray tube with copper anode at a tension of 35 kV. and a current of 40 ma. The window of aluminium foil was 30μ , and the mica slip for the hanging drop 0.02–0.025 mm. in thickness. The X-ray intensity at the distance of the hanging drop (3.5 cm.) was about 95,000 r./min. The dose was always the same, namely 2×10^6 r.

Irradiation media. The effect of irradiation under the above-mentioned circumstances on 0.1% erythrocyte suspensions in saline (0.9% NaCl solution) was always fixation ('saline effect', s.e.), but in a medium of 1 vol.