Fumaric Acid Oxidation by Ground Preparations of Pigeon Brain

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(Received 8 January 1945)

Fumarate catalysis has been extensively studied since it was first shown that small amounts of fumaric and other C_4 -dicarboxylic acids markedly increased the O_2 consumption of finely minced pigeon breast muscle (Gözszy & Szent-Györgyi, 1934; Annau, Banga, Gözszy, Huszak, Laki, Straub & Szent-Györgyi, 1935). These subsequent investigations have shown the effect to occur with many other tissues, and it is now generally believed that the catalysis is largely exerted on the pyruvate oxidation system (Annau, Banga, Blazso, Brukner, Laki, Straub & Szent-Györgyi, 1936; Annau & Erdős, 1939).

The present work has its origin in the observation of Banga, Ochoa & Peters (1939) that addition of fumarate to ground preparations of pigeon brain, respiring in a suitable medium, markedly stimulated both the O_2 uptake and pyruvate utilization. A repetition of these experiments, the details of which will be published later, has confirmed the increased O_2 uptake, but a slower rate of pyruvate disappearance has repeatedly been found. It seemed probable that this latter result might be due to the simultaneous oxidation of fumarate to pyruvate, even though this reaction does not take place in the presence of the *minced* tissue (Long, Ochoa & Peters, 1939). The work to be described shows clearly that this conversion can take place aerobically in *ground* preparations of pigeon brain, when the respiration medium contains arsenite to inhibit further oxidation of the accumulated pyruvate. Under these conditions, the activity of the fumarate oxidation system is catalyzed by inorganic phosphate and strongly inhibited by malonate, while adenine nucleotide is without effect. Observations made under anaerobic conditions, while differing in some details, have essentially confirmed these findings. A few experiments have also been carried out using *l*-malate in place of fumarate, with similar results.

EXPERIMENTAL

Materials

Fumaric and *l*-malic acids. The pure acids (Fraenkel), neutralized with NaOH (pH 7.3), were used. Final concentration, 0.005 M.

Oxaloacetic acid. Prepared from *dl*-malic acid (Fenton & Jones, 1900). For details of estimation, see text.

Adenine nucleotide. Three samples (Long, 1943) were used. Final concentration, 0.0003 M.

Sodium arsenite. An aqueous suspension of As_2O_3 (0.99 g.) was neutralized with 40% NaOH (pH 7.3) and diluted to 50 ml. This 0.2 M-arsenite solution was diluted as required to final concentration, 0.008 M.

Methods

(a) **Respiration technique.** Ground pigeon-brain preparations (suspensions), dialyzed against 0.4% KCl, were prepared as described by Long (1943); respiration was at 28° and pH 7.3 in a series of 50 ml. Erlenmeyer flasks. When O_2 uptakes were being measured, Barcroft flasks and manometers were used.

The enzyme suspension (1 ml., equivalent to 333 mg. fresh tissue) was added at zero time to the medium (1 ml.) containing fumarate, with or without arsenite, inorganic phosphate, adenine nucleotide, malonate, etc. After 40 min. respiration, the contents of the flasks were deproteinized with 25% trichloroacetic acid (1 ml.). Pyruvate was determined on measured fractions of the filtrate (Long, 1942).^{*} Inorganic phosphate was estimated as described by Long (1943).

(b) **Anaerobic procedure.** The dehydrogenases of pigeon brain were prepared according to Long (1943); some indication of their relative activities can be obtained from the data in Table 1, which shows the reduction rate of methylene blue under the conditions stated.

Table 1. *Methylene-blue reduction by different substrates, using a dehydrogenase preparation from pigeon brain*

(Each Thunberg tube contained 1.0 ml. enzyme suspension (equivalent to 150 mg. fresh brain tissue), 0.2 ml. 0.5 M-phosphate buffer, pH 7.3, and 0.2 ml. 0.02% methylene blue (added from hollow stopper at zero time). Total volume 2.0 ml. Reduction at 28° *in vacuo*.)

Substrate	Reduction time <i>t</i> (min.)	Activity (100/ <i>t</i>)
Nil	182	0.5
Fumarate	18½	5.4
<i>l</i> -Malate	12	8.3
Succinate	27½	3.6
α -Ketoglutarate	15	6.7
Pyruvate	16½	6.1
α -Ketobutyrate	18½	5.4

Since with fumarate or *l*-malate as H-donor, quite a visible amount of methylene blue remains in the oxidized form when equilibrium is reached, the end-point was taken

^{*} As shown below (p. 147), the results obtained by this method include oxaloacetate as well as pyruvate. Results described as those for pyruvate in the present paper should be interpreted accordingly.

after 90% reduction, as judged by the depth of colour of a control tube containing 10% of the initial methylene blue.

The experiment of Table 1 indicates that the enzyme preparation contains appreciable amounts of fumarase and *l*-malic dehydrogenase, in addition to the α -keto-acid dehydrogenase for which the method of preparation was originally designed.

Results

The effect of fumarate and l-malate on the O₂ uptake of ground and of minced preparations of pigeon brain. Long *et al.* (1939), presenting evidence against the theory that pyruvate is oxidized by minced pigeon brain through a succinic or citric acid cycle, observed that the rate of O₂ uptake of such a tissue preparation was not increased by the addition of fumarate or *l*-malate. A repetition of this work, using ground instead of minced brain, however, has led to totally different results, the respiration in this case being, markedly increased by the addition of 0.005M-fumarate (Table 2). It is probable that these results are due to the difference in permeability of the two pigeon-brain preparations towards fumarate.

Table 2. *The O₂ uptake of ground and of minced pigeon brain in the presence and absence of fumarate and l-malate*

(O₂ uptakes expressed as μ l./g. fresh tissue/hr. (average of closely agreeing duplicates). 0.05M-phosphate buffer, pH 7.3, present.)

Substrate	Exp.	Dialyzed brain suspension		Washed minced brain	
		O ₂ uptake at 28°		O ₂ uptake at 38°	
		Dialysis (hr.)	With residual substrate	Residual	With substrate
Fumarate	1	3	225	387	—
	2	6	166	293	—
	3	8	117	252	—
	4	—	—	261	270
<i>l</i> -Malate	5	—	—	252	261

The conversion of fumarate to pyruvate by dialyzed suspensions of pigeon brain, respiring in the presence of varying amounts of arsenite. The increased O₂ uptake observed following the addition of fumarate to a dialyzed suspension of pigeon brain, respiring in a phosphate medium, is accompanied by the formation of only a very small amount of pyruvate. If arsenite be also present in the medium, however, the accumulation of pyruvate is greatly increased, since under these conditions its removal from the system by further oxidation is arrested. The effect of varying concentrations of arsenite on the production of pyruvate is shown in Fig. 1.

It will be observed that maximal pyruvate production is obtained in the presence of 0.002M-arsenite, higher concentrations up to 0.008M being

without further effect. This observation is significant since it indicates that between these two concentrations arsenite, while inhibiting pyruvate oxidation completely, has no effect on the fumarate oxidation system, i.e. fumarase and *l*-malic dehydrogenase, unless both concentrations and those intermediate inhibit to the same extent, which is very unlikely. The literature contains two references to the effect of arsenite on the *l*-malic dehydrogenase

Fig. 1. *The effect of arsenite concentration on the aerobic oxidation of fumarate to pyruvate.* Pyruvic acid expressed as μ mol./333 mg. fresh tissue/40 min.

activity of an extract from pigs' heart. Green (1936) found that 0.03M-arsenite accelerated the oxidation of *l*-malate by 38%, while Das (1937a) reported that 0.01M-arsenite inhibited the enzyme by 77%. These two observations are difficult to reconcile, but do not conflict with the present work since the arsenite concentrations used are somewhat higher than those employed here. Thus there seems to be no evidence that arsenite has any effect on the oxidation of fumarate under the conditions described.

The effect of inorganic phosphate on the oxidation of fumarate by ground preparations of pigeon brain. One of the most striking observations concerning the oxidation of fumarate and *l*-malate by ground preparations of pigeon brain is the dependence of the reaction rate on the concentration of inorganic phosphate in the medium. This is shown under both aerobic and anaerobic conditions.

With the Thunberg technique and methylene blue as H-acceptor, the results shown in Table 3 were obtained. It will be observed that the activity of

the enzyme system is increased 4–12 times by the presence of 0.05M-phosphate. In the absence of fumarate, phosphate does not appreciably accelerate the residual reduction rate of methylene blue.

As the phosphate concentration was varied in these experiments, the osmotic pressure and Na⁺/K⁺ ratio of the media were kept constant, isotonic phosphate and chloride being interchanged. The residual phosphate concentration of the tissue preparation in the total volume was equivalent to about 0.00007M.

Table 3. *The effect of inorganic phosphate on the anaerobic oxidation of fumarate and l-malate*

(Experimental details as in Table 1.)

Substrate	Exp.	Activity (100/t)		Ratio of activities
		No phosphate added	0.05M-phosphate added	
Fumarate	1	0.9	3.7	4.1
	2	1.6	6.3	3.9
	3	0.5	6.1	12.2
	4	0.5	4.3	8.6
l-Malate	5	1.2	5.6	4.7
	6	1.4	6.3	4.5

Fig. 2. The effect of inorganic phosphate concentration on the activity of the anaerobic system: dehydrogenase preparation (≡ 150 mg. fresh brain tissue), 0.005M-fumarate and methylene blue (0.002%).

Fig. 2 shows the effect of increasing phosphate concentration on the rate of anaerobic oxidation of fumarate. The initial portion of the curve is very steep and maximal activity of the system is reached in the presence of 0.01M-phosphate; a slight decrease in activity is observed at higher phosphate concentrations.

Under aerobic conditions, pyruvate formation from fumarate is similarly dependent on the phosphate concentration of the medium. With 0.008M-arsenite present, the conversion of fumarate to pyruvate by dialyzed pigeon-brain suspensions in the presence and absence of added 0.05M-inorganic phosphate is shown in Table 4. The residual concentration of phosphate in the tissue preparation averaged 0.001M (cf. 0.00007M for the dehydrogenase preparation).

Table 4. *The effect of inorganic phosphate on the formation of pyruvate from fumarate*

Exp.	Pyruvic acid formed (μmol./g./hr.)	
	No phosphate added	0.05M-phosphate added
1	1.9	4.9
2	2.7	8.2

Fig. 3. The effect of inorganic phosphate concentration on the aerobic oxidation of fumarate to pyruvate. 0.008M-arsenite present.

Fig. 3 shows the pyruvate production at different phosphate concentrations. The curve differs from that obtained under anaerobic conditions, however, in that the well-defined peak at 0.01M-phosphate concentration is absent.

The effect of arsenate on the oxidation of fumarate. To a very limited extent, phosphate can be replaced by arsenate in the fumarate oxidation system. Table 5 shows the effect of increasing arsenate concentration on the conversion of fumarate to pyruvate by dialyzed pigeon-brain suspensions respiring in the presence of 0.008M-arsenite.

Table 5. *The effect of arsenate on fumarate oxidation*

(Inorganic phosphate absent.)

Arsenate concentration (M)	Pyruvic acid formed ($\mu\text{mol./g./hr.}$)
—	2.6
0.001	2.9
0.005	3.6
0.010	3.3
0.030	3.1
0.050	2.7

Maximal activity of the fumarate oxidation system is seen to be obtained when the arsenate concentration is about 0.005M, higher concentrations being less effective.

The effect of malonate on the fumarate oxidation system. Malonate is well known to inhibit both succinate oxidation and fumarate catalysis. Its effect on the oxidation of fumarate was first investigated under anaerobic conditions. Two typical experiments, in which varying malonate concentrations were used, are shown in Table 6.

Table 6. *The effect of malonate on the anaerobic oxidation of fumarate*

(Experimental conditions as in Table 1.)

Exp.	Malonate concentration (M)	Activity (100/t)
1	—	6.1
	0.0062	6.8
	0.0125	5.8
	0.0250	4.0
2	—	4.9
	0.0031	7.3
	0.0062	6.7
	0.0125	5.5
	0.0250	4.4

It will be seen that concentrations of malonate less than about 0.01M activate the anaerobic oxidation of fumarate, while higher concentrations cause inhibition. In the absence of fumarate, malonate had no effect on the activity of the residual system.

A study of the effect of malonate on the aerobic conversion of fumarate to pyruvate by dialyzed pigeon-brain suspensions in the presence of arsenite gave results of an entirely different nature. Under these conditions all concentrations of malonate inhibited the oxidation process, complete inhibition being observed in the presence of 0.03M-malonate (Table 7).

Table 7. *The effect of malonate on the aerobic oxidation of fumarate*

Malonate concentration (M)	Pyruvic acid formed ($\mu\text{mol./g./hr.}$)	Inhibition (%)
—	6.2	—
0.0005	6.1	2
0.0010	5.8	6
0.0020	5.1	17
0.0050	4.5	28
0.0100	2.8	55
0.0200	1.4	78
0.0300	0.0	100

These results may be compared with those obtained by Green (1936), who found that 0.03 and 0.3M-malonate inhibited the O_2 uptake of the *l*-malic dehydrogenase system from pigs' heart in the presence of *l*-malate, KCN, coenzyme I and methylene blue, to the extent of 11 and 47% respectively. Das (1937b), under comparable conditions, observed 50% inhibition of the activity of a *l*-malic dehydrogenase preparation by 0.06M-malonate. Thus it appears that at equivalent concentration, malonate is a more efficient inhibitor of the fumarate oxidation system in dialyzed pigeon-brain suspensions than of the reconstituted systems of Green and of Das.

Fig. 4. The time course of the aerobic oxidation of malate (curve A) and fumarate (curve B) to pyruvate. ••••• adenylypyrophosphate (0.0003M) present; ○○ adenylypyrophosphate absent.

The oxidation of fumarate and of l-malate in the presence and absence of adenine nucleotide. The two curves in Fig. 4 indicate that adenine nucleotide is without effect on the oxidation of fumarate and of

l-malate to pyruvate by dialyzed suspensions of pigeon brain in the presence of 0.05M-phosphate and 0.008M-arsenite. This fact is of interest in view of the observation of Banga *et al.* (1939), that adenine nucleotide markedly increased the rate of O₂ uptake of such a tissue preparation respiring in a medium consisting of phosphate, pyruvate and fumarate. Its importance will be made clear in a later paper in which the mechanism of this latter reaction is quantitatively studied.

A comparison of the curves in Fig. 4 shows also that *l*-malate is oxidized more rapidly than fumarate in the initial stages of the reaction, and that the rates later become equal. This is interpreted as indicating that the conversion of fumarate to *l*-malate must precede oxidation, but that in the later stages of the experiment the fumarase activity is sufficiently great to make the *l*-malic dehydrogenase activity the limiting factor in the rate of oxidation.

Pyruvate or oxaloacetate as the end-product of fumarate oxidation by respiring suspensions of pigeon brain. It has been assumed in the foregoing work that the oxidation of fumarate by molecular O₂ in the presence of dialyzed pigeon-brain suspensions involves the following sequence of reactions:

- (a) Fumarate → *l*-malate.
- (b) *l*-Malate → oxaloacetate.
- (c) Oxaloacetate → pyruvate.

In view of the likelihood that reaction (c) takes place to a limited extent only, it would be desirable to estimate the separate amounts of oxaloacetate and pyruvate produced, their sum being a function of the quantity of fumarate oxidized. This has involved an investigation of the colorimetric determination of oxaloacetate by the 2:4-dinitrophenylhydrazone method used for pyruvate (Long, 1942). As a result, it was found that oxaloacetate and pyruvate, at equivalent molecular concentration, give the same colour intensity. Thus the method, as applied to the protein-free filtrate from the respiration medium, yields the sum of the quantities of pyruvate and oxaloacetate formed, an amount which is proportional to the quantity of fumarate oxidized.

The sample of oxaloacetic acid, prepared as described under *Reagents*, contained about 20% unchanged *dl*-malic acid. This is shown by the fact that the CO₂ evolution from the β-keto-acid grouping, by aniline citrate, corresponds to only 80% of the theoretical, while the mol. wt. determination, by titration of the —COOH groups with alkali, agrees very nearly with the calculated value.

6.50 mg. required 0.891 ml. 0.1014N-NaOH. Assuming two —COOH groups, mol. wt., found = 130.7; calculated for oxaloacetic acid = 132; calculated for *dl*-malic acid = 134.

1.56 mg. in 2 ml. 0.1N-H₂SO₄, on treatment with aniline citrate, evolved 213 μl. CO₂ at N.T.P.; calculated for oxaloacetic acid = 265 μl. Purity = 80.3%.

The following experiment was carried out to estimate oxaloacetic acid by the colorimetric method of Long (1942). A series of pyruvic acid concentrations and two amounts of oxaloacetic acid were determined simultaneously. The oxaloacetic acid was corrected for its 19.7% *dl*-malic acid content, and found to give the same colour intensity as an equimolecular amount of pyruvic acid. The results are shown in Table 8.

Table 8. *The colorimetric determination of oxaloacetic acid*

Drum reading	Oxaloacetic acid (μmol.)		Pyruvic acid at equivalent drum reading (μmol.)
	Taken	Corrected for impurity	
0.443	0.382	0.306	0.310
0.566	0.509	0.408	0.400

The values given in Table 8 show that oxaloacetate is estimated as if it were pyruvate, the colorimetric method being unable to distinguish between the two. Hence the quantity hitherto estimated as pyruvate in protein-free filtrates is the sum of the amounts of pyruvate and oxaloacetate formed from fumarate, a figure which truly represents the extent of fumarate oxidation.

SUMMARY

1. The addition of small amounts of fumarate to a dialyzed pigeon-brain suspension, respiring *in vitro*, caused an increased O₂ uptake, an effect which is not observed with respiring minced brain tissue. In the presence of arsenite, pyruvic acid is formed in amounts which could be determined quantitatively.

2. The pyruvic acid formation under these conditions is catalyzed by inorganic phosphate, strongly inhibited by malonate, and unaffected by adenine nucleotide.

3. Under anaerobic conditions, with methylene blue as H-acceptor, essentially similar results have been obtained, although very low concentrations of malonate were found to be somewhat catalytic.

4. In so far as it has been studied, *l*-malate behaves like fumarate.

The author wishes to thank Prof. D. C. Harrison for his continued interest in the work.

REFERENCES

- Annau, E., Banga, I., Blazso, A., Brukner, V., Laki, K., Straub, F. B. & Szent-Györgyi, A. (1936). *Hoppe-Seyl. Z.* **244**, 105.
- Annau, E., Banga, I., Gözsy, B., Huszak, S., Laki, K., Straub, F. B. & Szent-Györgyi, A. (1935). *Hoppe-Seyl. Z.* **236**, 1.
- Annau, E. & Erdős, T. (1939). *Hoppe-Seyl. Z.* **257**, 111.
- Banga, I., Ochoa, S. & Peters, R. A. (1939). *Biochem. J.* **33**, 1980.
- Das, N. B. (1937*a*). *Biochem. J.* **31**, 1116.
- Das, N. B. (1937*b*). *Biochem. J.* **31**, 1124.
- Fenton, H. J. H. & Jones, H. O. (1900). *J. chem. Soc.* **77**, 69.
- Gözsy, B. & Szent-Györgyi, A. (1934). *Hoppe-Seyl. Z.* **224**, 1.
- Green, D. E. (1936). *Biochem. J.* **30**, 2095.
- Long, C. (1942). *Biochem. J.* **36**, 807.
- Long, C. (1943). *Biochem. J.* **37**, 215.
- Long, C., Ochoa, S. & Peters, R. A. (1939). *J. Physiol.* **96**, 7*P*.

Properties of Azide-catalase

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(Received 8 January 1945)

It is now well established that catalase is a conjugated protein with protohaematin as its prosthetic or active group. Catalase resembles acid methaemoglobin in its colour, its absorption spectrum, and above all in its property of combining reversibly and forming well-defined derivatives with HCN, H₂S, HN₃, HF, NH₂OH, NO and C₂H₅O.OH. However, it differs from methaemoglobin in three important properties, namely: (1) the colour and absorption spectrum of its compound with azide (NaN₃), (2) the reaction with H₂O₂, which consists in a violent, almost explosive, decomposition of the latter, during which it is impossible to observe any changes in colour and absorption spectrum of the enzyme, and (3) the stability of its trivalent iron which is not reduced even by Na₂S₂O₄. In this latter respect catalase differs fundamentally from all other haematin derivatives (Keilin & Hartree, 1936).

The main object of this investigation was to examine the properties of azide-catalase in the hope of gaining further insight into the mechanism of the reaction catalyzed by free catalase.

MATERIALS AND METHODS

Catalase used for this investigation was prepared from horse liver. The initial stages of extraction are based upon those of Zeile & Hellström (1930). They have previously been described by us (Keilin & Hartree, 1936, 1938), but we have since introduced slight modifications.

5.5 kg. liver is finely minced, mixed with an equal volume of tap water and allowed to stand overnight at 10–15°.

Next day, the fluid is pressed out and treated with 400 ml.* 90% ethanol/l. of fluid. The mixture is centrifuged for 30 min. and the red-brown supernatant fluid again precipitated by the addition of 200 ml.* 90% ethanol plus 100 ml. chloroform/l. When the resulting precipitate is centrifuged down a clear yellow-brown fluid is obtained with a green fluorescence. Examination of a 3 cm. layer with a small dispersion spectroscope should reveal the absorption band of catalase at 622 mμ. The fluid is left overnight at 5° when a brown precipitate containing much inactive protein is deposited. This is filtered off to give about 4 l. of a clear green-brown solution. The enzyme was removed from this solution by shaking it with three successive lots of 200 ml. calcium phosphate gel (a suspension containing 1 g. tricalcium phosphate/40 ml.), the pH being maintained at 5.6 by means of acetic acid. The combined cakes of the gel were suspended in water, brought to pH 5.6 and centrifuged. The fluid was discarded and the catalase was eluted by suspending the gel in water, adjusting the pH to 8.0 with dilute ammonia and centrifuging. Two further extractions were made with 0.02*N*-ammonia. The combined eluates (790 ml.) were brought to pH 5.5 and diluted to 1 l. Addition of 250 g. (NH₄)₂SO₄ yielded a precipitate of ferritin plus a little catalase. This was filtered off and the bulk of the catalase precipitated by addition of a further 100 g. (NH₄)₂SO₄. This precipitate was filtered off, dissolved in 50 ml. water and electro-dialyzed against running tap water to remove (NH₄)₂SO₄.

At this stage the catalase is pure enough for most purposes but can be purified further as follows: The solution is diluted to 250 ml. and treated with 50 ml. calcium phosphate gel at pH 7.0 which is centrifuged off and discarded; it is then treated with 150 ml. calcium phosphate gel in three lots at pH 6.4. The gel is centrifuged off, washed once with water and extracted five times with 0.025*M*-Na₂HPO₄ (total

* The optimal volumes of ethanol should be determined by preliminary small-scale experiments.