THE OXIDATION OF L-AMINO ACIDS BY MITOCHONDRIA FROM RAT LIVER

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SUMMARY

The oxidation of L-alanine by actively phosphorylating mitochondria from rat liver has been shown. L-Alanine alone was oxidised very slowly, but, in the presence of T.C.A. intermediates and L-glutamate, there was a marked stimulation of its oxidation. The system was very responsive to low concentrations of L-alanine and to most of the T.C.A. intermediates. Of the T.C.A. intermediates α-oxo-glutarate showed the greatest stimulation. L-Alanine and L-aspartate reduced the conversion of DL-[1-^{14}C]-glutamate to carbon-dioxide. It is concluded that the probable mechanism of oxidation is not by action of L-amino acid oxidase but via transamination to α-oxo-glutarate and subsequent oxidation of the pyruvate produced. The results give evidence for mitochondrial L-glutamic dehydrogenase and transaminase as a functional cyclic system in the deamination of amino acids and as mediators of the oxidation of their carbon skeletons.

INTRODUCTION

It is known that animals have the means to deaminate amino acids and oxidize their carbon skeletons, but apart from special cases like glutamic acid little is known about the enzymic mechanisms generally involved. From the existing evidence two such enzymic mechanisms appear to be possibilities. These are the reactions catalysed by the L-amino acid oxidase of BLANCHARD, GREEN, NOCITO AND RATNER[1] and the transdeamination reactions suggested by BRAUNSTEIN[2,3] and again by ROWSELL[4]. The former system has been studied in isolation from the cell and as such is not very active. The latter system is currently being investigated by KLYUGE[5] in dispersions of liver and by BÄSSLER AND HAMMAR[6] in cell fractions of liver.

HIRD AND ROWSELL[7] found that mitochondria from liver of the rat were capable of carrying out a general reversible transamination reaction between glutamic acid and a number of other amino acids and ROWSELL[4,8] has subsequently considerably extended these observations. Mitochondria from rat liver, kidney and heart are thus established as a site of deamination. The now accepted function of mitochondria as the site of oxidation and decarboxylation of pyruvate and α-oxo-glutarate suggested to us that other α-oxo acids might be oxidized by mitochondria with the resultant metabolism of the acyl-coenzyme A derivatives.

Abbreviation: T.C.A., Tricarbonic acid.

Accordingly we decided to investigate mitochondria as a system for the deamination and oxidation of some appropriate amino acids. The present paper is concerned chiefly with L-alanine.

MATERIALS AND METHODS

Materials

Hexokinase was either prepared by the method of BERGER, SLEIN, COLOWICK AND CORI[9] or purchased from the Sigma Chemical Co. Generally labelled L-[^{14}C]-alanine and DL-[1-^{14}C]glutamic acid were obtained from the Radiochemical Centre, Amersham, England.

Mitochondrial preparations

Mitochondria were prepared essentially by the method of ALDRIDGE[11] excepting that 0.27 *M* sucrose was used. The mitochondrial pellet prepared from 5 g of liver was resuspended in 0.27 *M* sucrose. Prepared in this manner they were able to oxidize pyruvate, octanoate, glutamate, α-oxo-glutarate and succinate and were able to carry out oxidative phosphorylation. Mitochondrial protein, estimated by the method of ALDRIDGE[11] was between 2 and 7 mg/flask.

Medium

All flasks contained 0.4 ml of mitochondrial preparation in 0.27 *M* sucrose together with the following substances at these final concentrations: 30 m*M* potassium phosphate buffer pH 7.4, 1 m*M* ethylenediaminetetraacetate, 13 m*M* KCl, 26 m*M* TRIS buffer pH 7.4, 2 m*M* ATP, 5 m*M* $MgCl_2$, 40 m*M* glucose. Glucose and 25 K-M units of hexokinase were added in 0.2 ml from the side arm. The final volume in the flask was 2.5 ml, with a final pH of 7.4. The gas phase was air and the $^{14}CO_2$ was collected in the centre well in 0.2 ml of 2 *N* NaOH.

Estimation of $^{14}CO_2$

The alkali containing the absorbed $^{14}CO_2$ from the centre well was washed out and diluted with a suitable amount of sodium carbonate. The carbonate was precipitated and plated on paper discs (Whatman No. 3) using the method described by ARONOFF[10]. The $Ba^{14}CO_3$ was counted at infinite thickness to 1% accuracy. Results are expressed as counts/min/5 mg of mitochondrial protein.

RESULTS

Transamination between L-alanine and L-glutamate

The method used to show reversible transamination reactions between L-alanine and L-glutamate was similar to that used by HIRD AND ROWSELL[7]. The results were similar to that observed earlier and were repeated here only to determine the existence of transaminase activity in actively phosphorylating mitochondria prepared in the present way.

The oxidation of generally labelled L-alanine by rat-liver mitochondria

Table I shows that a range of concentrations of L-alanine alone did not significantly increase the respiratory activity of mitochondria over the low level of the

TABLE I

OXIDATION OF GENERALLY LABELLED L-[^{14}C]ALANINE BY MITOCHONDRIA

Results expressed as QO_2 (μl/mg protein/h) and as $^{14}CO_2$ produced/5 mg mitochondrial protein. Incubated for 1 h under conditions described in text. Counted at infinite thickness, $3.6 \cdot 10^4$ counts/min calculated to infinite thickness added to each flask. Results corrected to a standard specific activity.

Substrate	*QO_2*	*Counts/min*
Control (no substrate)	6.1	—
0.5 m*M* L-alanine	6.1	270
1 m*M* L-alanine	6.1	172
5 m*M* L-alanine	5.3	245
10 m*M* L-alanine	6.4	298
20 m*M* L-alanine	5.6	380

control without any addition. This suggests that L-amino acid oxidase activity of the mitochondrial preparation is either absent or of a very low order. The percentage conversion of L-alanine to carbon-dioxide is quite small and would not be observed as an increase in oxygen consumption. From the results given later with T.C.A. intermediates it is very likely that the metabolism observed results from a stimulation by small amounts of endogenous substrates associated with the mitochondria.

Fig. 1 shows that α-oxo-glutarate is able to increase 18-fold the appearance of $^{14}CO_2$ from generally labelled L-alanine (10 m*M*) and that a maximum stimulation is reached at about 10 m*M*. Oxidation of L-alanine alone was found to be variable but was at all times less than in the presence of 0.1 m*M* α-oxo-glutarate. The system is very responsive to small changes in concentration, more than half of the total activity resulting from a concentration of α-oxo-glutarate of only 1 m*M*.

Fig. 2 shows that with the α-oxo-glutarate at about saturation level (10 m*M*) the rate of oxidation of L-alanine increases with its concentration to a maximum above 20 m*M* L-alanine. A time progress curve of this reaction at two concentrations

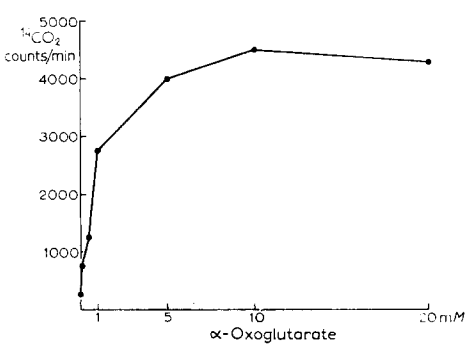

Fig. 1. The oxidation of generally labelled L-[^{14}C]alanine (10 m*M*) in the presence of varying concentrations of α-oxo-glutarate. Incubated for 1 h under conditions described in text. Counted at infinite thickness and corrected to 5 mg mitochondrial protein; $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

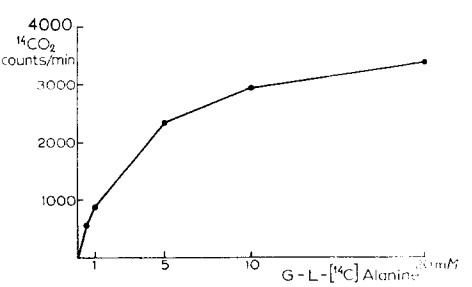

Fig. 2. The oxidation of varying concentrations of generally labelled L-[^{14}C]alanine in presence of α-oxo-glutarate (10 m*M*). Incubated for 1 h under conditions described in text. Counted at infinite thickness, $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask and results calculated to standard specific activities/5 mg mitochondrial protein.

of α-oxo-glutarate can be seen in Fig. 3. This shows that the stimulation of the oxidation of alanine persists over a long period.

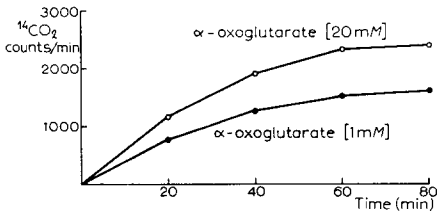

Fig. 3. Time progress curve for the oxidation of generally labelled L-[^{14}C]alanine (20 mM) in the presence of α-oxo-glutarate at 1 and 20 mM. Incubation for 1 h under conditions described in text. Counted at infinite thickness and corrected to 5 mg mitochondrial protein, $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

The effect of washing the mitochondria on the system oxidising L-*alanine*

In order to determine whether the oxidation system was firmly bound, a mitochondrial preparation was repeatedly washed, whilst another portion of the same preparation was subjected to the same manipulations, but without washing. Table II shows the results of this experiment. There was no significant difference in activity of the much-washed preparation over the control preparation. It is concluded that the system oxidizing L-alanine is firmly bound to mitochondria and does not have, as an essential component, a contaminant from some other cell fraction.

TABLE II

THE EFFECT OF REPEATED WASHING ON THE OXIDATION OF GENERALLY LABELLED L-[^{14}C]ALANINE SYSTEM

The effect of repeated washing of mitochondria with 0.27 M sucrose on the oxidation of generally labelled L-[^{14}C]-alanine. Incubated for 1 h under conditions described in text. Counts made at infinite thickness and corrected to 5 mg mitochondrial protein, $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

	Mitochondria	
	Twice washed Counts/min	*Six times washed Counts/min*
10 mM generally labelled L-[^{14}C]alanine	160	215
10 mM generally labelled L-[^{14}C]alanine + 1 mM α-Oxo-glutarate	2380	2655
10 mM generally labelled L-[^{14}C]alanine + 5 mM α-Oxo-glutarate	4960	4770
10 mM generally labelled L-[^{14}C]alanine + 10 mM α-Oxo-glutarate	5145	4900

The oxidation of L-*alanine in the presence of pyruvate*

Low concentrations of pyruvate added to the system result in a considerable stimulation of $^{14}CO_2$ production from generally labelled L-alanine. Fig. 4 shows the nature of the stimulation over a short time interval (10 min), as it is related to the concentration of pyruvate. The inference which is drawn from this result is that transamination allows an exchange of amino groups between the labelled L-alanine

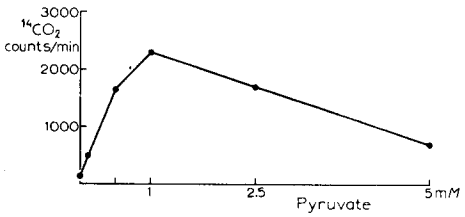

Fig. 4. Oxidation of generally labelled L-[^{14}C]alanine in the presence of varying concentrations of pyruvate. Incubation for 10 min under conditions described in text. Counted at infinite thickness and calculated to 5 mg mitochondrial protein. $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, added to each flask.

and the non-labelled pyruvate. The graphs shows that with concentrations of pyruvate greater than 1 m*M* there is an abrupt fall in the amount of $^{14}CO_2$ which is formed. This fall is due, most likely, to dilution with unlabelled pyruvate.

The oxidation of L-*alanine in the presence of other metabolites related to the T.C.A. cycle*

Glutamate added to the system also stimulates the formation of $^{14}CO_2$ from generally labelled L-alanine. This is consistent with the oxidation of the L-glutamate by L-glutamic dehydrogenase to form α-oxo-glutarate; this enzyme is known to be present on mitochondria. The α-oxo-glutarate so formed is then available for transamination reactions with L-alanine. Fig. 5 shows the relationship of L-glutamate concentration to the oxidation of L-alanine. The remaining graphs in Fig. 5 show that citrate, isocitrate, succinate, fumarate and malate also stimulate the oxidation of L-alanine and that their effects differ in detail. However, none were as efficient as α-oxo-glutarate or pyruvate. These results have been obtained with mitochondria from different rats, so that precise quantitative comparisons are not possible.

With citrate, succinate and glutamate there is a fall in each case in the amount of $^{14}CO_2$ which appears in the presence of the higher concentrations of the substrates

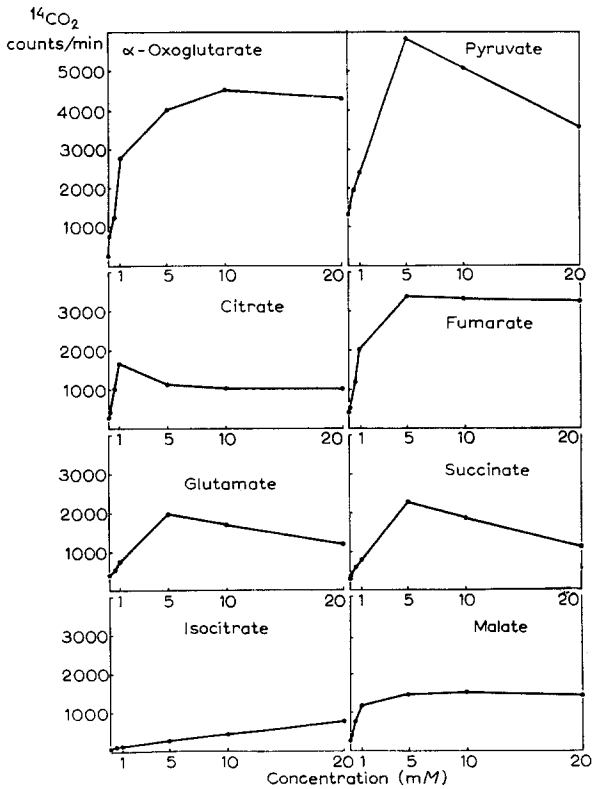

Fig. 5. Oxidation of generally labelled L-[^{14}C]alanine in the presence of varying concentrations of α-oxo-glutarate, citrate, L-glutamate, D-isocitrate, pyruvate, fumarate, succinate and L-malate. Incubated for 1 h in medium described in text. Counted at infinite thickness and corrected to 5 mg mitochondrial protein, $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

tested. This fall can be explained in terms of dilution, as in the case of the oxidation of labelled L-alanine in the presence of unlabelled pyruvate. In the case of L-glutamate, there is the further possibility of the reverse transamination reactions due to high concentrations of glutamate.

Comparison of the effect of α-oxo-glutarate and L-malate on the oxidation of L-alanine

Stimulation of the oxidation of labelled L-alanine by T.C.A. intermediates can be attributed to the supply of oxalacetate (to accept acetyl groups) α-oxo-glutarate (to accept amino groups), from any pyruvate formed or from a combination of these. A comparison between the stimulating effect of oxalacetate and α-oxo-glutarate would therefore provide useful information about the rate-limiting step involved. However, samples of oxalacetate tested showed the presence of interfering proportions of pyruvate. Malate was then used as a substitute for oxalacetate. It can be seen from the time progress curve (Fig. 6) that, over the whole period studied, α-oxo-glutarate was the more active stimulator. Indeed α-oxo-glutarate consistently showed a greater effect than any other T.C.A. intermediate (Fig. 5). However, such an effect would also be shown if there were less dilution of labelled by unlabelled compound in the case of α-oxo-glutarate than of the other T.C.A. intermediates. In this type of experiment the superiority of α-oxo-glutarate over the other intermediate is therefore only consistent with, but not proof of, a primary role as amino-group acceptor.

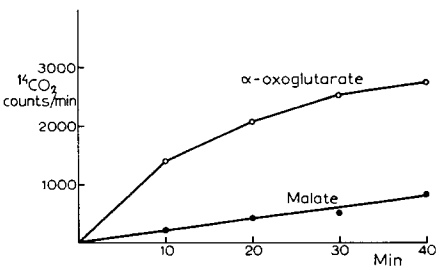

Fig. 6. Time progress curve for the oxidation of generally labelled L-[^{14}C]alanine (10 mM) in the presence of α-oxo-glutarate (1 mM) and L-malate (1 mM). Incubated under conditions described in text. Counts made at infinite thickness, $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

The effect of L-alanine and L-aspartate on the decarboxylation of [1-^{14}C]α-oxo-glutarate derived from DL-[1-^{14}C]glutamate

The immediate product of the oxidation of glutamic acid in the system is α-oxo-glutarate, which may then be oxidized and decarboxylated, with carbon one of glutamate appearing as carbon-dioxide. However, in the presence of a system carrying out transamination reactions between L-alanine and L-aspartate on the one hand and α-oxo-glutarate on the other, there should be a conversion of α-oxo-glutarate back

TABLE III

THE OXIDATION OF DL-[^{14}C]GLUTAMATE IN THE PRESENCE OF L-ALANINE AND L-ASPARTATE

The effect of L-alanine, L-aspartate on the conversion of DL-[1-^{14}C]glutamate (1 mM) to $^{14}CO_2$. Incubated for 1 h under conditions described in text. Malonate (20 mM) present in each flask. Counted at infinite thickness approximately $1.5 \cdot 10^4$ counts/min, calculated at infinite thickness, added to each flask.

	Counts/min	*Inhibition*
1 mM DL-[1-^{14}C]glutamate	1586	—
1 mM DL-[1-^{14}C]glutamate + 10 mM L-alanine	945	41 %
1 mM DL-[1-^{14}C]glutamate + 10 mM L-aspartate	1056	29 %

to glutamate. The overall effect of such transaminating reactions would then be seen as a reduction in carbon-dioxide derived from carbon one of glutamate. Table III shows that the presence of L-alanine and L-aspartate does in fact have this effect. Malonate was added to reduce possible complications following the further metabolism of succinate to oxalacetate.

DISCUSSION

The deamination of L-alanine may proceed either by the action of L-amino acid oxidase or by transaminases, both processes forming the same products. In systems as complex as the T.C.A. cycle the factors influencing the conversion of each of the carbon atoms of L-alanine through pyruvate to carbon-dioxide are therefore not easy to assess. If the mechanism of deamination were by the action of L-amino acid oxidase, then any T.C.A. intermediate giving rise to oxalacetate would stimulate the oxidation of the pyruvate produced. Also any T.C.A. intermediate producing α-oxo-glutarate, pyruvate or oxalacetate may act as acceptors of amino groups and therefore as a means of converting L-alanine to pyruvate. Further any unlabelled T.C.A. intermediate added to the system may cause a dilution of the labelled pyruvate or more probably the labelled T.C.A. intermediates produced from pyruvate. Such dilution would be related to the extent to which the products of the metabolism of pyruvate mix with the added intermediates. This effect would be observed as an inhibition and it is likely that even the observed stimulation of the oxidation of L-alanine, by T.C.A. intermediates, contains such a component. This effect would be more pronounced with increase in concentration of the diluent and is probably the explanation of the linear fall in the rate of oxidation of L-alanine seen in certain cases in Fig. 5.

It is clear that precise interpretation of results is not possible, as the above factors may be operating simultaneously. An unequivocal description of the mechanism of deamination is not, therefore, possible. However, the following evidence is consistent with an explanation in terms of transamination.

1. The oxygen consumption in the presence of L-alanine alone has been shown to be no different from that of the control mitochondria without added substrates. BÄSSLER AND HAMMAR[6] have also shown that there is no increase in the respiratory rate of mitochondria using L-valine, L-isoleucine and L-leucine and our unpublished results with these amino acids are in agreement with theirs. ROWSELL[8] reaches a similar conclusion with respect to the oxidation of several amino acids by "particle" preparations from rat liver. These results suggest either the absence of L-amino acid oxidase or its quantitative unimportance.

2. Mitochondrial preparations from rat liver have been shown to perform reversible transamination reactions between L-alanine and L-glutamate (HIRD AND ROWSELL[7], ROWSELL[8]).

3. α-Oxo-glutarate has been found to be the most effective stimulator of the oxidation of L-alanine. Such stimulations are observed over a range of concentrations and over the complete time course investigated.

4. Pyruvate has been found to be a potent stimulator. Such a stimulation is only consistent with a mechanism by transamination.

5. L-Glutamate and the T.C.A. intermediates, all of which stimulate the oxidation of L-alanine, can be regarded as precursors of α-oxo-glutarate. KLYUGE[5], working

with homogenates of rat liver, also gives evidence for the importance of α-oxo-glutarate in the transfer of amino groups from L-alanine to citrulline.

6. When glutamate is oxidised by the mitochondrial preparation, the production of carbon-dioxide from carbon atom one of glutamate is inhibited by L-alanine and L-aspartate. This observation is consistent with transamination of the α-oxo-glutarate formed to glutamate. That the observed inhibition does not result from dilution of the α-oxo-glutarate, by metabolism of pyruvate, is suggested by the presence of this inhibitory effect when malonate is in the medium.

Mitochondria have been shown by BÄSSLER AND HAMMAR[6] to have increased respiration in the presence of α-oxo-butyrate, α-oxo-valerate and α-oxo-isovalerate. In experiments to be reported in a later paper we have obtained conversion of generally labelled L-[^{14}C]valine, L-isoleucine, L-leucine to $^{14}CO_2$ by mitochondria from liver and kidney tissue of the rat. Thus there is good evidence that oxidation of the α-oxo acids, produced by deamination of amino acids, can be performed by mitochondria.

BÄSSLER AND HAMMAR[6] also present evidence, based on oxygen consumption, that concurrent transamination and oxidation of amino acids are catalysed by mitochondria from rat liver.

From the evidence now available, it seems highly probable that the mechanism of deamination of alanine by mitochondria from rat liver is by transamination and that the amino group acceptor is α-oxo-glutarate. Such a reaction represents a cycle involving the L-glutamic dehydrogenase and transaminase(s) of mitochondria as enzymes in this cycle. This conclusion has not been yet definitely established to cover other amino acids, but it has been observed in preliminary experiments that mitochondria from rat kidney show a considerable stimulation of the conversion of generally labelled L-[^{14}C]valine, isoleucine and L-leucine to carbon-dioxide, when α-oxo-glutarate is added to the system.

NOTE ADDED IN PROOF

STILL *et al.*[12], using a particulate "cyclophorase" preparation from kidney, showed a stimulation of oxygen uptake with L-alanine in the presence of α-oxo-glutarate and other TCA intermediates.

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