THE METABOLISM OF ASCARIS LUMBRICOIDES OVARIRES

III. THE SYNTHESIS OF ALANINE FROM PYRUVATE AND AMMONIA*

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Summary

Small amounts of α-amino nitrogen are formed when dialysed ovary homogenates are incubated with ammonium chloride, sodium pyruvate, and various cofactors.

Under anaerobic conditions chloramphenicol and to a lesser degree ascorbic acid and large amounts of lactate enhance the reductive amination of pyruvate. The reaction is dependent on the presence of reduced diphosphopyridine nucleotide and adenosine triphosphate. Hydrogen acceptors such as methylene blue considerably decrease alanine formation.

In the presence of chloramphenicol and the necessary cofactors the optimum pH for the formation of alanine from pyruvate and ammonium chloride is 7.2; this is in contrast to the two pH optima (6.9 and 8.2) obtained for this system in the absence of chloramphenicol. A progress curve of the amination reaction was constructed.

The significance of the reductive amination of pyruvate in the formation of amino acids in the ovaries of Ascaris lumbricoides is considered.

I. INTRODUCTION

Ascaris lumbricoides is a parasitic roundworm commonly found in pigs. A production of over 200,000 eggs per day by female worms has been recorded (Brown and Cort 1927) and hence the ovaries of this worm provide good material for the study of amino acid and protein metabolism.

Earlier work (Pollak and Fairbairn 1955a, 1955b) demonstrated a relatively high alanine content in the non-protein fraction of the ovaries, low glutamic acid dehydrogenase activity, and a very high alanine–glutamic acid transaminase activity. A non-specific alanine-α-keto acid transaminase was also detected, while the corresponding glutamic acid-α-keto acid transaminase could not be demonstrated (Pollak, unpublished data). These facts, together with the evidence of α-amino nitrogen formation by ovary homogenates in the presence of pyruvate and ammonium chloride (Pollak and Fairbairn 1955b), indicate that alanine rather than glutamic acid plays the key role in amino acid metabolism. This concept is further strengthened by the fact that A. lumbricoides is a facultative anaerobe with a high rate of aerobic and anaerobic glycolysis and thus pyruvate would be readily available, and in addition large amounts of ammonia are present in the ovary tissue (Pollak and Fairbairn 1955a, Pollak 1957).

In a previous study of the amino acid metabolism of ovary homogenates of A. lumbricoides (Pollak and Fairbairn 1955b) it was demonstrated that although

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reductive amination of pyruvate occurred in this tissue, only small yields of alanine were obtained, and in several experiments the ovary preparations were altogether inactive for this reaction. However, since the reductive amination of pyruvate may well play a central part in the amino acid metabolism of the ovaries of *A. lumbricoides*, the system has now been studied in greater detail. The present investigation concerns factors which enhance the synthesis of alanine from pyruvate and ammonium chloride.

II. MATERIALS AND METHODS

The ovaries were isolated from female *A. lumbricoides* which had been collected in 1 per cent. saline at 37°C, at Homebush State Abattoirs, Sydney. The maintenance of the worms *in vitro* and the dissection of the ovaries were described by Pollak and Fairbairn (1955a).

Between 1 and 2 g of tissue were homogenized in 6 ml of a cold 0·5 per cent. KCl solution containing 12·2 mg nicotinamide per ml. To the homogenate an equal volume of M/10 Sorensen phosphate buffer (pH 6·9 or 7·2) was added and the suspension dialysed with stirring in the cold (2°C) against 2 l. distilled water for 6–8 hr.

All experiments were carried out under anaerobic conditions in evacuated Thunberg tubes. Essentially the same results were obtained when the tubes were alternatively evacuated and gassed three times with a mixture of 95 per cent. nitrogen and 5 per cent. carbon dioxide. The tubes were incubated at 37°C. After incubation the reaction mixture (3 ml) was deproteinized by the addition of 7 ml of ice-cold ethanol. The protein precipitate was separated by centrifugation and the alcoholic extract made up to 10 ml; 0·3-ml samples were withdrawn for the measurement of total α-amino nitrogen and 5 ml was used for qualitative and quantitative amino acid chromatography as previously described (Pollak and Fairbairn 1955a, 1955b). α-Amino nitrogen was determined by measuring the optical density of the ninhydrin compound at 570 μm in a Beckman model DU spectrophotometer. In all experiments alanine made up by far the greatest proportion of the synthesized α-amino nitrogen, therefore alanine was used to prepare the standard curve for the photometric determinations. For the measurement of total α-amino nitrogen special precautions were necessary to ensure complete removal of ammonia from the solution, as this compound also reacts with the ninhydrin reagent and thus interferes with the determination. After the addition of 0·1 ml of 0·1N NaOH, each 0·3-ml sample was left in a vacuum dessicator overnight over conc. H₂SO₄, at a pressure of less than 14 mm Hg. Controls included with each experiment ensured that all the ammonia was removed. Another precaution had to be taken whenever chloramphenicol was present in the reaction mixture. Chloramphenicol itself reacts with the ninhydrin reagent, resulting in a high optical density at 570 μm. In this case blanks (reaction mixtures at 0 min) containing chloramphenicol with and without other components were run with every experiment to allow for the optical density attained by chloramphenicol in the absence and presence of substrates.

All substrates were obtained from commercial sources, neutralized whenever necessary, and used in concentrations as indicated in the text or in the legends of the tables and figures.
III. Results

*a*-Amino Nitrogen Formation

In the early experiments of this investigation it was confirmed that ovary homogenates are able to synthesize *a*-amino nitrogen in the presence of large concentrations of pyruvate and ammonium ions. The yield of *a*-amino nitrogen formed was always small and not significantly affected by the addition of adenosine triphosphate (ATP) and reduced diphosphopyridine nucleotide (DPNH) (Table 1). These

### Table 1

**α-AMINO NITROGEN FORMATION BY OVARY HOMOGENATES OF ASCARIS LUMBRICOIDES**

Each tube contained 2 ml of a 10 per cent. ovary homogenate (pH 6.9); after the addition of all the components the volume was made up to 3.0 ml with 0.1M phosphate buffer (pH 6.9). Temperature, 37°C; gas phase, vacuum; duration of experiment, 3 hr. ATP, adenosine triphosphate, DPNH, reduced diphosphopyridine nucleotide

<table>
<thead>
<tr>
<th>Age of Worms</th>
<th>Additions</th>
<th>Quantity (µmoles)</th>
<th>Number of Experiments</th>
<th>α-Amino Nitrogen (µmoles/g tissue/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>Ammonium chloride</td>
<td>120</td>
<td>5</td>
<td>2.2*</td>
</tr>
<tr>
<td></td>
<td>Sodium pyruvate</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>Ammonium chloride</td>
<td>120</td>
<td>5</td>
<td>2.1*</td>
</tr>
<tr>
<td></td>
<td>Sodium pyruvate</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>Ammonium chloride</td>
<td>80</td>
<td>2</td>
<td>0.7</td>
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<tr>
<td></td>
<td>Sodium pyruvate</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>Ammonium chloride</td>
<td>40</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Sodium pyruvate</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>Ammonium chloride</td>
<td>120</td>
<td>4</td>
<td>7.0†</td>
</tr>
<tr>
<td></td>
<td>Sodium pyruvate</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>Ammonium chloride</td>
<td>120</td>
<td>4</td>
<td>6.4†</td>
</tr>
<tr>
<td></td>
<td>Sodium pyruvate</td>
<td>120</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DPNH</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>Ammonium chloride</td>
<td>120</td>
<td>2</td>
<td>6.9†</td>
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<td>Sodium pyruvate</td>
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</tr>
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<td></td>
<td>ATP</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DPNH</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* In three other experiments no amination took place.
† In one other experiment no amination took place.
experiments demonstrated only small α-amino nitrogen production, but several consistent features were revealed. Amination occurred only when the substrates (pyruvate and ammonium ions) were present in large quantities. Ovary homogenates which were prepared from worms that had been kept up to three days in an inorganic medium (Baldwin and Moyle 1947) gave rise to greater amounts of α-amino nitrogen than did homogenates prepared from fresh worms (Table 1). A pH activity curve in the presence of phosphate buffer showed that the reaction exhibited two pH optima (Fig. 1).

![Fig. 1](image)

Fig. 1.—The effect of pH on α-amino nitrogen formation in ovary homogenates. Reaction mixture: 1·5 ml homogenate in 0·5 per cent. potassium chloride containing nicotinamide, 120 μmoles ammonium chloride, 120 μmoles sodium pyruvate, and 1 ml 0·1M phosphate buffer of required pH to bring the volume to 3·0 ml. Other conditions as cited in the text.

(b) Enhancement of Alanine Formation

In an attempt to enhance the rate of pyruvate reduction to form alanine, chloramphenicol and ascorbic acid were added to the reaction mixture. Both compounds have been used previously for the selective channelling of particular pathways of pyruvate reduction (Groth and LePage 1954). Table 2 demonstrates the effect of chloramphenicol and ascorbic acid on the rate of alanine formation from pyruvate and ammonium ions.

Chloramphenicol produced a three-fold increase, while ascorbic acid caused only a small increase in alanine formation. Chloramphenicol and ascorbic acid together caused the production of a greater amount of alanine than the addition of either one of the compounds; however, no true additive effect was obtained. Furthermore, in the presence of chloramphenicol, alanine accounted for practically all the newly formed α-amino nitrogen, with small increases in the glutamic acid concentration, while in the absence of chloramphenicol, alanine as well as aspartic acid was formed (Pollak and Fairbairn 1955b).

In the absence of chloramphenicol the addition of ATP and DPNH had no effect on the amination reaction (Table 1) but in the presence of chloramphenicol considerable increases in alanine formation were observed (Table 2). When DPNH was replaced by a hydrogen acceptor such as methylene blue, alanine formation
was greatly decreased (Table 2), indicating the dependence of reductive amination of pyruvate on a hydrogen donor such as reduced coenzyme and therefore upon anaerobic conditions.

**Table 2**

**EFFECT OF CHLORAMPHENICOL AND ASCORBIC ACID ON ALANINE FORMATION**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Quantity (μmoles)</th>
<th>Alanine Formed* (μmoles/g tissue/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>8·0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>15</td>
<td>15·3</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>30</td>
<td>13·8</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>15</td>
<td>17·3</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>15</td>
<td>62·0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>4</td>
<td>106·2</td>
</tr>
<tr>
<td>DPNH</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>15</td>
<td>17·3</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Methylene blue</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*Mean values of three experiments.

In the absence of either pyruvate or ammonium ions or in the absence of both these compounds, chloramphenicol did not give rise to alanine, or any other form of α-amino nitrogen; this was confirmed in every instance by means of paper chromatography.

Chloramphenicol and ascorbic acid are thought to act by shifting the balance of the electron transport system of pyruvate reduction, so that one particular hydrogen...
acceptor is favoured (Groth and LePage 1954). Therefore the addition of an end-product of pyruvate reduction other than alanine should have a similar effect, as the equilibrium of that particular reaction would be changed in such a way as to favour other pathways of pyruvate reduction. This is shown in Table 3, where the $\alpha$-amino nitrogen produced by the addition of a large amount of lithium lactate is compared with the amount of $\alpha$-amino nitrogen produced in the presence of ascorbic acid. The results indicate that the end-product of pyruvate reduction to lactate, and ascorbic acid which may act as an inhibitor of this reaction (Groth and LePage 1954), have the same enhancing effect on alanine formation.

**Table 3**

**EFFECT OF LACTATE AND ASCORBIC ACID ON $\alpha$-AMINO NITROGEN FORMATION**

Each tube contained 1·5 ml of a 10 per cent. ovary homogenate (pH 6·9), 120 $\mu$moles ammonium chloride, and additions as shown in the table. The volume was made up to 3·0 ml with 0·1M phosphate buffer (pH 6·9). Temperature 37°C; gas phase, vacuum; duration of experiment, 1 hr.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Quantity ($\mu$moles)</th>
<th>$\alpha$-Amino Nitrogen ($\mu$moles/g tissue/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pyruvate</td>
<td>120</td>
<td>7·0</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>120</td>
<td>13·0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>120</td>
<td>12·1</td>
</tr>
<tr>
<td>Lithium lactate</td>
<td>120</td>
<td>0·5</td>
</tr>
</tbody>
</table>

(c) **Effect of pH**

In the presence of chloramphenicol and other cofactors the pH curve gave a single sharp peak with an optimal pH at 7·2 (Fig. 2). This is in contrast to the pH activity curve obtained under conditions cited in Figure 1.

(d) **Time Curve of Alanine Formation**

The time curve of the reaction was followed in a dialysed homogenate with the necessary additions for optimal activity. So far all attempts to purify the enzyme responsible for the reductive amination of pyruvate have failed. Therefore no kinetic studies or rate measurements have been carried out apart from the simple progress curve of alanine formation by a dialysed homogenate (Fig. 3).
IV. DISCUSSION

The mechanism of amino acid formation from α-keto acids has been under discussion for some considerable time. The earliest experiments stem from the school led by von Euler, who showed that in microorganisms the reverse reaction of glutamic dehydrogenase enabled them to utilize ammonium ions for α-amino nitrogen synthesis (Adler et al. 1938). In 1939, Krebs and Cohen showed that a similar reaction accounted for amination of α-ketoglutaric acid in animal tissues. For many years this reaction was considered as the only pathway of α-amino nitrogen formation. In 1947, Kritzman indicated that pyruvate and oxalacetate were also
involved in amination reactions. Further experiments with animal tissues (Wiss 1948; Canzanelli, Rapport, and Guild 1950; Daugherty 1954) indicated that pyruvate might be involved in direct amination. However, contradictory results concerning the cofactor requirement of the enzyme involved and the aerobic or anaerobic nature of the reaction made the interpretation of these results difficult.

It has now been established that alanine can serve as an amino group donor in many transamination reactions (Rowsell 1951; Rudman and Meister 1953), this function was formerly ascribed only to glutamic acid and aspartic acid. Hence the reduction of pyruvate to form alanine may now be regarded as an important avenue of amino acid synthesis. Furthermore in organisms in which there is a considerable excess of glycolysis over aerobic respiration, the accumulating three-carbon compound will be of greater importance as an amino group acceptor than \(\alpha\)-ketoglutarate.

The results of the preliminary experiments (Table 1, Fig. 1), particularly the pH activity curve which exhibited two pH optima, indicated either a multiple step reaction or a competition between two or more enzymes for the same substrate (pyruvate) causing the trough in the pH activity curve. Of great interest, also, was the observation that homogenates prepared from worms which had been kept in the inorganic non-nutrient medium for 3 days were more active than homogenates prepared from fresh ovary tissue. The ammonium ion concentration of ovary tissue is known to decrease on aging the worms in such a non-nutrient medium (Pollak 1957). The enhancement of alanine formation in homogenates of aged worms could be explained either by the formation of activating substances for alanine formation or by the production of depressors of competing reactions. In the latter instance, such a compound can act as a metabolic regulator, accelerating alanine formation from pyruvate at the expense of other reduction reactions of pyruvate.

In a recent study on the metabolism of pyruvate of rat liver and tumour homogenates, Groth and LePage (1954) showed that ascorbic acid, glutathione, and chloramphenicol act as such-metabolic regulators determining the reductive pathway of pyruvate. In the present investigation glutathione was considered to be unsuitable, since quantitative paper chromatography was used for the determination of the amino acids. But ascorbic acid, which in rat tissue had a similar effect to glutathione, and chloramphenicol were used in an attempt to enhance the amination of pyruvate. Chloramphenicol was the more effective agent, and, in further experiments in the presence of this compound, the reductive amination of pyruvate was strongly dependent on DPNH and ATP. A similar diphosphopyridine (DPN) and DPNH dependency has been demonstrated by Wiame and Pierard (1955) for a reversible and specific alanine dehydrogenase system in *Bacillus subtilis*. In another investigation on the amination of pyruvate in *Aerobacter aerogenes*, Fowler and Workman (1955) stipulated the formation of a phosphorylated intermediate.

In the presence of chloramphenicol the optimal pH is 7.2 (Fig. 2) compared with the two maxima shown in Figure 1 (6.9 and 8.2) in the absence of chloramphenicol. These results support the concept that a competitive reaction, retarded by the presence of chloramphenicol, causes the depression in the pH curve.

The reductive amination of pyruvate not only provides the ovary tissue with alanine but, since an active non-specific alanine-\(\alpha\)-keto acid transaminase is present
in this tissue (Pollak, unpublished data), a whole array of amino acids may be produced. Furthermore not only is α-amino nitrogen produced, but DPNH is re-oxidized in the process and DPN becomes available for other biological oxidations. As *A. lumbricoides*, along with most parasitic nematodes, is a facultative anaerobe and as there is an excess of pyruvate production over pyruvate oxidation, this substrate may take the part which α-ketoglutaric acid usually plays in aerobic organisms.

The comparison of the effect of lactic acid and ascorbic acid on the reductive amination of pyruvate (Table 3) proved to be very instructive. The results indicate that large amounts of lactate had the same enhancing effect on pyruvate reduction to alanine as had the addition of ascorbic acid. This is in agreement with the evidence of Groth and LePage (1954) who found that ascorbic acid inhibited the reduction of pyruvate to lactate.

This reduction of pyruvate to alanine, dependent on DPNH and ATP, seems to compete with other systems for substrate molecules, and chloramphenicol, ascorbic acid, and excess of lactate all enhance the formation of alanine. The removal of the newly formed alanine, and thus the maintenance of the reaction, seems to be assured by the presence of the non-specific alanine–α-keto acid transaminase as well as by the specific and active alanine–glutamic acid transaminase. Such activity is demonstrated by the small increases of the amino acid spots on the chromatograms. Alanine–glutamic acid transaminase favours glutamic acid formation 2 : 1 over alanine formation from glutamic acid under conditions very similar to those of the present experiments (Pollak and Fairbairn 1955b). This equilibrium towards glutamic acid formation, as well as the dialysis procedure which was routinely carried out, exclude the possible formation of alanine from a coupling of the glutamic acid dehydrogenase and alanine–glutamic acid transaminase systems.

The recent work of Wiame and Pierard (1955) showed that in *B. subtilis* oxidative deamination of aspartate, glutamate, and glycine proceeds via a transamination reaction to pyruvate, followed by the deamination of the newly formed alanine. Since the reaction is reversible in this organism there also is a pathway for direct alanine synthesis. Evidence pointing to direct alanine formation has also been obtained by Fairhurst, King, and Sewell (1956) who used 16 different bacterial strains and species.

In the ovary tissue of *A. lumbricoides* the high rate of glycolysis (Rathbone and Rees 1954), the high ammonia content (Pollak and Fairbairn 1955a), and the demonstration that ammonia, far from being an excretory product, is utilized *in vivo* for α-amino nitrogen formation (Pollak 1957) as well as the results obtained in the present investigation on the amination of pyruvate, all indicate that pyruvate may well hold a key position for the formation of amino acids in this organism.

V. Acknowledgment

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VI. References


