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# LACTIC AND SUCCINIC ACIDS AS EXCRETORY PRODUCTS OF *POLYMORPHUS MINUTUS* (ACANTHOCEPHALA) *IN VITRO*

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## INTRODUCTION

When adult worms of the acanthocephalan parasite, *Polymorphus minutus*, are incubated for 4 hr. at $41 \cdot 7 \pm 0 \cdot 5°$ C. under near-anaerobic conditions in a balanced salt solution containing radioactive glucose, they excrete two substances which contain approximately equal amounts of radioactivity. It was suspected that these metabolites might be organic acids. They were found to be non-volatile, dialysable, free from phosphorus and amino groups and not carbohydrate in nature. Examination of the incubation medium by one-dimensional, descending paper chromatography in a Shandon Panglass 300 Chromatatank indicated that one of the metabolites was lactic acid. The solvent systems used were *PrF* and *EtAm* (Smith, 1960), and the organic acids were located by dipping dry papers in the bromcresol green reagent described by Smith. The other metabolite appeared to be succinic acid with the *PrF* solvent system, but its position on the paper after the *EtAm* solvent did not correspond exactly with that of authentic succinic acid, although it was always in the same region of the paper as succinic, malic and fumaric acids. The aim, therefore, of the work described in this paper was to identify, by means of gas chromatography, the unknown organic acid and to confirm the identity of the lactic acid. The results are considered to be applicable to the carbohydrate metabolism of the worms *in vivo*, because care was taken to ensure that the worms remained healthy during the incubation period.

## MATERIALS AND METHODS

*Incubation procedure.* Worms of known age, sex and wet weight were obtained from freshly killed Khaki Campbell ducks which had been fed on BOCM* baby chick crumbs. They were kept in glucose-free, physiological saline (Hanks, 1948), at $41 \cdot 7 \pm 0 \cdot 5°$ C., pH 7·8 and in equilibrium with air for less than an hour before being exposed to radioactive glucose. Crystapen benzylpenicillin and Streptomycin sulphate were present in the saline in concentrations of 1 mg./ml. For experimental purposes the worms were transferred from these conditions to a sterile glass vessel containing a known amount of sterile Hanks's saline and antibiotics, and D-[U-$^{14}$C]glucose. Nitrogen was bubbled through the vessel for 15 min. before the taps were closed and

* British Oil Cake Manufacturers.

the worms were left for a further 3¾ hr. The worms were then removed and the incubation medium was analysed for radioactive excretory products.

It was presumed, for three reasons, that the worms remained in a healthy state during the incubation period. First, the oxygen tension in the incubation vessel was measured with an oxygen electrode and found to be about 10 mm. Hg. This value, and those of the temperature and pH in the experiments, are similar to measurements made in the small intestine of ducks by Crompton, Shrimpton & Silver (1965) and Crompton (1966). Secondly, the worms were observed to move spontaneously for 19 days under these conditions provided that glucose was present in the concentration originally prescribed by Hanks (1948). Thirdly, it was found that worms which had been kept for 4 hr. *in vitro* under the incubation conditions could be returned by surgical procedures to the small intestine of a duck where their development continued. This test was performed once with seven worms, of which four were found to be attached to their host's intestinal wall 24 hr. after the operation. The establishment of the other three worms could have failed owing to the presence of anaesthetics which are known to affect schistosomes (Dickerson, 1965).

The possibility that the radioactive metabolites had been produced by bacteria in the medium was also investigated. No bacteria could be seen in a drop of medium examined at the end of the incubation period. A tenth of a ml. of the incubation medium and successive 20-fold dilutions of it were inoculated on to plates of a reinforced clostridial medium and placed under anaerobic conditions at 37° C. *Clostridia* are common bacteria of the vertebrate intestine, but no growth was visible on any of the plates after a fortnight.

The results presented in this paper were obtained from the analysis of the incubation medium involving thirteen male and thirteen female 5-day-old worms weighing 13 mg. The worms were placed in 1·5 ml. of Hanks's saline containing 0·33 mg. D-[U-$^{14}$C]glucose/ml. of specific activity 16·2 $\mu$c./mg. Only two radioactive metabolites were detected by paper chromatography, and these were the only ones present on four other occasions when worms varying in age from 1 to 30 days old were used. All the radioactive chemicals employed in this work came from The Radiochemical Centre, Amersham, Bucks, U.K.

*Extraction of organic acids.* 7 ml. of N-HCl were added to about 0·2 ml. of the incubation medium, and the resulting solution was extracted continuously for 7 hr. with 50 ml. diethyl ether. The ethereal phase was dried overnight with anhydrous $Na_2SO_4$. After the $Na_2SO_4$ had been removed by filtration through a No. 1 sintered glass funnel, the volume of ether was reduced by distillation to about 1 ml. An air condenser, acting as a fractionating column, was fitted to the distillation flask; this modification was necessary to minimize the loss of radioactivity during the removal of the ether.

*Preparation of methyl esters.* Two methods were used to prepare the methyl esters of the extracted organic acids. In the first, the final ml. of ether from the organic extract was distilled to dryness, then 2·0 ml. of dry methanol, containing 2% (v/v) $H_2SO_4$ (sp. gr. 1·84) were added and the mixture was refluxed for 1 hr. Next, $NaHCO_3$ was added until effervescence ceased, leaving the solution ready for analysis by gas chromatography. In the second method, 0·1 ml. methanol was added to the final ml. of ether (Schlenk & Gellerman, 1960), and diazomethane from a microgenerator (Roper & Ma, 1957) was bubbled through the solution until a yellow colour persisted.

*Estimation of the radioactivity in the volatile fraction.* Half a millilitre of incubation medium was placed in a double-necked, 100 ml. flask with a tap funnel attached to one neck and a Dreschel head to the other. Air, under pressure, was used to force about 20 ml. N-HCl into the flask simultaneously with the bubbling of nitrogen through the Dreschel head. The air was switched off and the nitrogen passed for 10 min. through the acidic medium at 60° C. into an absorption tube containing 2 ml. ethanolamine and 4 ml. ethylene glycol monomethyl ether (Jeffay & Alvarez, 1961). Nitrogen was next passed for 5 min. through the acidic medium at 100° C. into a fresh absorption tube, and this process was repeated at 100° C. using another fresh absorption tube. Samples of 1 ml. each from the three absorption tubes were added to 5 ml. of a mixture of toluene-ethylene glycol monomethyl ether (2:1 v/v) containing 5·5 g./l. 2,5-diphenyl oxazole, and the radioactivity was counted in a Panax, A.C. 300/6, water-cooled, liquid scintillation counter standardized for this counting medium with *n*-[1-$^{14}$C]hexadecane.

*Gas chromatography.* The methyl esters of organic acids were chromatographed in the vapour phase using a Panchromatograph (W. G. Pye and Co. Ltd., Cambridge) fitted with a macro-argon detector. Glass columns 150 × 0·5 cm. diam., were used packed with one of two stationary phases; 10% (w/w) polyethyleneglycol adipate on Gas-Chrom Z 100–120 mesh (Applied Science Laboratories Inc., State College, Penn., U.S.A.) and 10% (w/w) Apiezon L grease on Gas-Chrom Z. 100–120 mesh. The polyethyleneglycol adipate column was operated at 100° C. for 4 min. after injection of a sample, then temperature programmed at 6° C./min. until 180° C. was reached. The carrier gas flow rate was maintained at a constant 70 ml./min. argon. The Apiezon L column was also operated at 100° C. for 4 min. after injection, then temperature programmed at 6° C./min. until 200° C. was reached. Carrier gas flow rate was maintained at 40 ml./min. throughout. The identity of specific components during gas chromatography was confirmed by the addition of internal markers of authentic methyl esters. Radioactive components were collected by attaching to the effluent of the gas chromatograph a column (35 × 1·1 cm. internal diameter) fitted with a No. 1 sintered glass disk at its lower end and containing 7 ml. toluene in which was dissolved 4 g./l. 2,5-diphenyl oxazole and 0·1 g./l. 1,4-bis-(5-phenyloxazole-2-yl) benzene. These solutions were counted in vials in the Panax scintillation counter as above.

## RESULTS

*Volatile fraction.* Table 1 shows the loss of radioactivity by volatilization of an aliquot of incubation medium (0·5 ml.) giving 73,900 disintegrations/sec. after heating with N-HCl first to 60° C. then cooling to room temperature followed by boiling then recooling and finally reboiling. Details are given in the Materials and Methods section.

The loss of radioactivity by volatilization after heating with HCl was found to be very small. The amount given off between room temperature and 60° C. is likely to be dissolved $CO_2$, while the fraction volatilized by boiling probably contains short-chain fatty acids. There does not appear to be any continued loss of radioactivity due to degradation of the main radioactive components after boiling with HCl as found by cooling and reboiling. The radioactivity volatilized by the second boiling was almost zero.

*Gas chromatography of incubation medium acids.* Preliminary results of the gas chromatography on polyethyleneglycol adipate of the methyl esters of the organic acid fraction showed that almost all the radioactivity was divided fairly equally between two peaks. The first of these two peaks was, as expected, identical in retention time with methyl lactate and the second with methyl succinate. A number of other acids of biological importance were chromatographed but most of these had considerably longer retention times as can be seen from Table 2.

Table 1. *Volatile fraction of incubation medium*

| Temperature (°C.) | Time of heating (min.) | Radioactivity volatilized disintegration/sec. | Radioactivity volatilized (%) |
|---|---|---|---|
| 60 | 10 | 470 | 0·64 |
| 100 | 5 | 762 | 1·03 |
| 100 | 5 | 24 | 0·03 |

Table 2. *Retention times of methyl esters of organic acids relative to methyl palmitate*

(The column was 150 × 0·5 cm. and packed with 10% (w/w) polyethyleneglycol adipate on Gas Chrom Z. The operating temperature was 180° C.)

| Ester | Retention time |
|---|---|
| Lactic | Too short to measure |
| Fumaric | 0·16 |
| Succinic | 0·19 |
| Maleic | 0·23 |
| Malic | 0·62 |
| Aconitic | 1·4 (tails) |
| Citric | 2·7 |
| Pyrazoline-3,4-dicarboxylic | 92 |

If attempts are made to form the methyl esters of fumaric and maleic acids with diazomethane the compound dimethyl pyrazoline-3,4-dicarboxylate (Loudon, 1957, *IVa*, p. 266) is formed. This was used as a useful means of effecting a better separation of fumaric, succinic and maleic acids on the gas chromatograph. Since all the radioactivity was eluted with the earliest peaks when the methyl esters from the incubation medium were chromatographed isothermally at 180° C., temperature programming was adopted. A very good separation of all the peaks in this mixture was obtained by operating the chromatograph at 100° C. for 4 min. after injection of the sample, followed by temperature programming at 6° C./min. Tables 3 and 4 show the results of gas chromatography of the methyl esters from the incubation medium on the two different stationary phases employed. The identity of the named peaks was ascertained by first chromatographing without, then with, internal markers.

*Incubations with horse-heart muscle preparation.* A preparation of horse heart-muscle was made as described by Keilin & Hartree (1938), and the final suspension was stored in 100 ml. 0·25 M-$Na_2HPO_4$ containing 200 g. sucrose. This preparation will contain a number of enzymes including succinic dehydrogenase and fumarase. Consequently, if succinic acid is incubated with this preparation most of it would be expected to be transformed into fumaric acid and malic acid. An experiment was therefore designed

to compare the action of the horse heart-muscle preparation on both [1-4-$^{14}C_2$]succinic acid and the incubation medium of *P. minutus*. Four 10 ml. test tubes were labelled and made up as in Table 5.

Table 3. *Gas chromatography of methyl esters of incubation medium on polyethyleneglycol adipate*

| Peak no. | Peak identity | Radioactivity (disintegrations/sec.) |
|---|---|---|
| 1 | — | 6 |
| 2 | Lactic | 185 |
| 3 | — | 8 |
| 4 | Succinic | 210 |
| 5 | — | 6 |
| 6 | Malic | 5 |
| 7 | — | 5 |
| 8 | Palmitic | 3 |
| 9 | — | 5 |
| 10 | Stearic | 9 |
| 11 | Oleic | 5 |
| | | Total 447 |

The amount of radioactivity injected was 507 disintegrations/sec. and the recovery from the chromatograph was therefore 88 %.

Table 4. *Gas chromatography of methyl esters of incubation medium on Apiezon L grease*

| Peak no. | Peak identity | Radioactivity (disintegrations/sec.) |
|---|---|---|
| 1 | — | 2 |
| 2 | — | 0 |
| 3 | — | 1 |
| 4 | Lactic | 60 |
| 5 | — | 4 |
| 6 | — | 2 |
| 7 | Succinic | 82 |
| 8 | — | 3 |
| 9 | — | 6 |
| 10 | Palmitic | 2 |
| 11 | — | 4 |
| 12 | Oleic | 3 |
| 13 | Stearic | 1 |
| 14 | — | 2 |
| | | Total 172 |

The amount of radioactivity injected was 202 disintegrations/sec. and the recovery from the chromatograph was therefore 84 %.

The tubes were incubated at 37° C. for 2 hr. in a water bath with air from a manifold bubbling slowly through their contents. The incubation was terminated by the addition of 1 ml. N-HCl to each tube and the organic acids were extracted by continuous ether extraction as described previously. The radioactive components of all the tubes except tube 2 were extracted quantitatively into the ether. In tube 2 only 41 % of the radioactivity was extracted into the ether, the remainder presumably being largely $^{14}C$-glucose. The other extracts were methylated with diazomethane and reduced to a volume suitable for gas chromatography. The methyl esters were injected successively

on to the gas chromatograph and the peaks were collected separately from the effluent into 7 ml. toluene scintillation fluid as before.

Tube 4, the blank tube, gave no radioactive peaks and the chromatogram showed a large peak of methyl succinate and a number of other smaller peaks some of which were recognizable as long-chain fatty acid esters. In tube 3, the control tube, the radioactivity was confined to the succinic acid peak. The pattern of peaks was similar

Table 5. *Incubation of horse heart-muscle preparation with* [1-4-$^{14}C_2$]*succinic acid and incubation medium of* Polymorphus minutus

| Tube 1<br>$^{14}C$-succinic acid | Tube 2<br>$^{14}C$-incubation medium | Tube 3<br>control | Tube 4<br>blank |
|---|---|---|---|
| 1 ml. 0·1 M-phosphate buffer pH 7·3 containing 2·4 mg. succinic acid | 1 ml. 0·1 M-phosphate buffer pH 7·3 containing 2·4 mg. succinic acid | 1 ml. 0·1 M-phosphate buffer pH 7·3 containing 2·4 mg. succinic acid | 1 ml. 0·1 M-phosphate buffer pH 7·3 containing 2·4 mg. succinic acid |
| 0·1 ml. water containing 10 $\mu$c. [1-4-$^{14}C_2$]succinic acid (48 $\mu$c./mg.) | 0·1 ml. incubation medium | 0·1 ml. water containing 10 $\mu$c. [1-4-$^{14}C_2$]succinic acid (48 $\mu$c./mg.) | 0·1 ml. water |
| 0·3 ml. cytochrome c (10 mg./ml.) in buffer | 0·3 ml. cytochrome c (10 mg./ml.) in buffer | 0·3 ml. cytochrome c (10 mg./ml.) in buffer | 0·3 ml. cytochrome c (10 mg./ml.) in buffer |
| 50 $\mu$l. buffer | 50 $\mu$l. buffer | 50 $\mu$l. malonic acid 0·2 g./ml. in buffer | 50 $\mu$l. buffer |
| 0·1 ml. horse heart prep. | 0·1 ml. horse heart prep. | 0·1 ml. horse heart prep. | 0·1 ml. horse heart prep. |

Table 6. *Comparison of the radioactive acids produced when horse heart-muscle preparation is incubated with* [1-4-$^{14}C_2$]*succinic acid and a similar component from the incubation medium of* Polymorphus minutus

| | Radioactive component at start of incubation | |
|---|---|---|
| Acids produced after incubation with horse heart prep. | [1-4-$^{14}C_2$]succinic acid<br>% | *Medium from *P. minutus*<br>% |
| Succinic | 3·4 | 35·5 |
| Malic | 54·6 | 36·7 |
| Citric | 3·0 | 0 |
| Pyrazoline dicarboxylic (fumaric) | 6·1 | 15·7 |
| Unknown | 22·8 | 0 |
| Total | 89·9 | 87·9 |

* For comparison, the radioactivity from lactic acid in this medium has been ignored.

to that of tube 4 except for an overload of methyl malonate. In tube 2 containing the methyl esters of the incubation medium, 88 % of the total radioactivity collected was in peaks identical with the esters of lactic, succinic, malic and pyrazoline-3-4-dicarboxylic acids. The latter would be formed from the action of diazomethane on fumaric acid (Loudon, 1957). In tube 1, which started with [1-4-$^{14}C_2$]succinic acid, the radioactivity was more scattered. Nevertheless, 90 % of the collected radioactivity appeared in peaks identical with the following esters: succinic, malic, citric, pyrazoline-3-4-dicarboxylic (fumaric) and an unknown compound. Table 6 compares the action of the horse heart particles on radioactive components of tubes 1 and 2.

From the results given in Table 6 it is clear that not so much of the component identical with succinic acid in the medium from *P. minutus* interacted with the horse

heart preparation enzymes as did [1-4-$^{14}C_2$]succinic acid. Nevertheless, most of the transformed radioactivity appeared in the peaks of malic and fumaric acids indicating that the component was indeed succinic acid.

## DISCUSSION

The identification of succinic acid among the excretory products of *Polymorphus minutus* provides the first record of the excretion of this organic acid by an acanthocephalan worm. *P. minutus* has also been found to excrete lactic acid as do *Moniliformis dubius* (Laurie, 1957) and *Neoechinorhynchus emydis* and *N. pseudemydis* (Dunagan, 1964). The fact that succinic acid has not been found to be an excretory product of *M. dubius* is of interest because Laurie (1959) extended his earlier work on this parasite and studied its aerobic metabolism. He found, after careful analysis of the incubation medium, that formic and acetic acids were excreted as well as lactic acid, but he makes no mention of succinic acid. It is possible that formic and acetic acids were present in the radioactive volatile material excreted by *P. minutus*. Graff (1964, 1965) showed that radioactive succinic, malic, lactic and fumaric acids can be detected in extracts made from whole *M. dubius*, which have been incubated with either $^{14}$C-glucose or $NaH^{14}CO_3$. Bryant & Nicholas (1965) also found these four radioactive acids were produced when homogenates of the body wall of *M. dubius* were incubated with $^{14}$C-glucose under conditions far removed from those *in vivo*. It is important, therefore, that the medium remaining after *M. dubius* has been incubated with exogenous carbohydrate under anaerobic conditions should be analysed for succinic acid. If *M. dubius* does not excrete succinic acid, in contrast to *P. minutus*, the intermediary metabolism of these parasites, from the same phylum and a similar environment, must be markedly different. The species of *Neoechinorhynchus*, studied by Dunagan, may also excrete succinic acid because he noted that the amounts of lactic acid excreted by the worms did not account for all the glycogen metabolized by them under starving conditions.

No evidence has been obtained for the formation of succinic acid by *P. minutus*. Von Brand (1966) has tabulated the helminths known to excrete succinic acid and discussed its production. Simpson & Awapara (1966) and Scheibel & Saz (1966) have studied mechanisms for the formation of succinic acid in marine molluscs and the cestode, *Hymenolepis diminuta*, respectively. Simpson & Awapara investigated the metabolism of $^{14}$C-glucose by preparations of the mantles of *Rangia cuneata*, *Crassostrea virginica* and *Volsella demissus*, and concluded that glucose is degraded to phosphoenolpyruvate which is carboxylated to form oxalacetate and thence to succinate. Scheibel & Saz, however, decided that the pathway for the conversion of $^{14}$C-glucose to succinate is through the carboxylation of pyruvate followed by reduction to succinate. It is probable that the production of succinic acid by *P. minutus* will eventually be shown to be along one of these pathways.

## SUMMARY

The acanthocephalan parasite, *Polymorphus minutus*, has been found to excrete radioactive lactic and succinic acid, and a small amount of radioactive, volatile material, when incubated with [U-$^{14}$C]-glucose under conditions designed to simulate those *in*

*vivo*. The acids were identified by paper and gas chromatography, and the identity of succinic acid was confirmed with an enzyme preparation.

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## REFERENCES

BRAND, VON T. (1966). *Biochemistry of Parasites*. New York and London: Academic Press.

BRYANT, C. & NICHOLAS, W. L. (1965). Intermediary metabolism in *Moniliformis dubius* (Acanthocephala). *Comp. Biochem. Physiol.* **15**, 103–12.

CROMPTON, D. W. T. (1966). Measurements of glucose and amino acid concentrations, temperature and pH in the habitat of *Polymorphus minutus* (Acanthocephala) in the intestine of domestic ducks. *J. exp. Biol.* **45**, 279–84.

CROMPTON, D. W. T., SHRIMPTON, D. H. & SILVER, I. A. (1965). Measurements of the oxygen tension in the lumen of the small intestine of the domestic duck. *J. exp. Biol.* **43**, 473–8.

DICKERSON, G. (1965). Effects of anaesthetics on mature infections of *Schistosoma mansoni* in the white mouse. *Nature, Lond.* **206**, 953–4.

DUNAGAN, T. (1964). Studies on the carbohydrate metabolism of *Neoechinorhynchus* spp (Acanthocephala). *Proc. helminth. Soc. Wash.* **31**, 166–72.

GRAFF, D. J. (1964). Metabolism of $C^{14}$-glucose by *Moniliformis dubius* (Acanthocephala). *J. Parasit.* **50**, 230–34.

GRAFF, D. J. (1965). The utilization of $C^{14}O_2$ in the production of acid metabolites by *Moniliformis dubius* (Acanthocephala). *J. Parasit.* **51**, 72–75.

HANKS, J. H. (1948). The longevity of chick tissue cultures without renewal of the medium. *J. cell. comp. Physiol.* **31**, 235–60.

JEFFAY, H. & ALVAREZ, J. (1961). Liquid scintillation counting of carbon-14. *Anal. Chem.* **33**, 612–15.

KEILIN, D. & HARTREE, E. F. (1938). Cytochrome oxidase. *Proc. R. Soc.* B **125**, 171–86.

LAURIE, J. S. (1957). The *in vitro* fermentation of carbohydrates by two species of cestode and one species of Acanthocephala. *Exptl Parasit.* **6**, 245–60.

LAURIE, J. S. (1959). Aerobic metabolism of *Moniliformis dubius* (Acanthocephala). *Exptl Parasit.* **8**, 188–97.

LOUDON, J. D. (1957). Compounds containing a five membered ring with two hetero atoms: pyrazole and iminazole groups. In *Chemistry of Carbon Compounds* (ed. E. H. Rodd). Amsterdam: Elsevier.

ROPER, R. & MA, T. S. (1957). Diazomethane as a reagent for microsynthesis. *Microchem. J.* **1**, 245–60.

SCHEIBEL, L. W. & SAZ, H. J. (1966). The pathway for anaerobic carbohydrate dissimilation in *Hymenolepis diminuta*. *Comp. Biochem. Physiol.* **18**, 151–62.

SCHLENK, H. & GELLERMAN, J. L. (1960). Esterification of fatty acids with diazomethane on a small scale. *Anal. Chem.* **32**, 1412–14.

SIMPSON, J. W. & AWAPARA, J. (1966). The pathway of glucose degradation in some invertebrates. *Comp. Biochem. Physiol.* **18**, 537–48.

SMITH, I. (1960). *Chromatographic and Electrophoretic Techniques I*. London: William Heinemann Medical Books, Ltd.