Some Acids Belonging to the Citric Acid Cycle in the Liver Fluke, *Fasciola hepatica*, L. *

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Mitochondria from vertebrates are claimed to be the sites for reactions concerned, *i.a.* in the citric acid cycle, the oxidation-reduction chain, and oxidative phosphorylation.[1] In *Fasciola hepatica* the mitochondria deviate morphologically from those of higher animals.[2,3] It thus seemed of interest to study some substances and systems involved in the mentioned reactions. This paper deals with a study of the citric cycle acids in homogenates from the liver fluke.

Living flukes were washed in 0.15 M NaCl and frozen at −80°C. They were stored at −20°C until used. For paperchromatographic studies of malic, fumaric, succinic, isocitric aconitic, and citric acids about 500 flukes (50 g wet weight) were ground with quartz and 10 % HPO_3 (250 ml). After centrifugation (3000 *g* for 10 min), the supernatant was extracted continuously for 12 h with diethyl-ether. The ether extract was then evaporated and to the brown residue 0.1−0.2 ml of distilled water was added; pH 7.0. The suspension obtained was centrifuged and the whole supernatant was used for two-dimensional chromatography on Whatman No. 1 paper with ethanol-ammonia-water and propanol-eucalyptol-formic acid as solvent systems.[4] The chromatograms were exposed in UV-light at 240−260 nm. (Mineralight V 41) and developed with bromocresol green [4] and *p*-dimethyl-aminobenzaldehyde.[5] The results are shown in Fig. 1. Some of the spots found were cut out and eluted with ether which was evaporated. The residue was treated with distilled water and the solution obtained rechromatographed in the basic or acid systems mentioned above.[4]

For a semiquantitative determination of the acids found in the fluke, 50−200 μg portions of the mentioned acids were analyzed according to the described technic and the areas of the spots were compared. The fluke homogenate was also directly tested for malic acid with malic dehydrogenase [6] and for citric acid by transformation into pentabromoacetone.[7] The results are shown in Table 1.

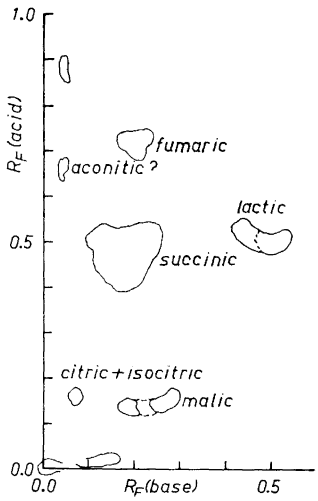

Fig. 1. Two-dimensional paper chromatography of some acids in *Fasciola hepatica*.

For the demonstration of keto acids, 25−50 g wet weight liver fluke were homogenized and deproteinized as earlier described. After centrifugation at 3000 *g* for 10 min at 0°C the supernatant was diluted 10-fold with distilled water and 0.4 ml freshly prepared 0.5 % dinitrophenyl hydrazine in 6 M HCl per 100 ml solution were added. After 20 min at + 38°C, the hydrazones were extracted several times with ether.[8] The extracts obtained were further treated as described in Hais' and Maceks handbook,[9] but instead of ethyl acetate, ether was used. The final extracts were evaporated almost to dryness

Table 1. Semiquantitative determination of citric acid cycle acids in liver fluke. The values are calculated per 100 g liver fluke, wet weight.

Citric acid	≈	200 μg*
Aconitic acid ?	<	100 ,,
α-Keto glutaric acid	<	5 ,,
Succinic acid	>	400 ,,
Fumaric acid	≈	300 ,,
Malic acid	≈	200 ,,
Oxalacetic acid	<	5 ,,

* Supported by a grant from *Jordbrukets Forskningsråd*, Stockholm.

* Verified with the pentabromoacetone method.

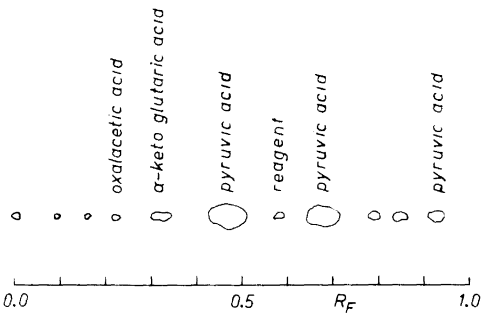

Fig. 2. One-dimensional paper chromatography of some hydrazones of keto acids in *Fasciola hepatica.*

under a fan and the hydrazones were taken up in a small volume of 0.2 M phosphate buffer of pH 6.2. This solution was applied to phosphate impregnated Whatman No. 3 paper and chromatographed in *tert*-amyl alcohol-ethanol-water (5:1:4).[10,11] The chromatograms were sprayed with 0.5 M NaOH.[9] The results are shown in Fig. 2.

Controls with known amounts of keto acids were run through the whole procedure and the size of the spots compared with those from the fluke homogenates. The semi-quantitative determination of the acids found are summarized in Table 1.

The results show that many of the common cycle acids are found in the liver fluke. The high amount of succinic acid is remarkable.

Organisms with mitochondria [1-3] and the ability to utilize O_2[13-15] might be supposed to have a citric acid cycle. At any rate, the liver fluke lives in an environment poor in O_2[16,17] and the utilization of glucose aerobically differs only slightly from anaerobic utilization.[15] These findings could be interpreted as signs that the fluke has a poorly developed citric acid cycle or lacks a complete cycle. The cycle acids found in the parasite could then mainly or completely have their origin in the host. If this is the case, then the high concentration of succinic acid ought to be due to an accumulation since in the fluke it dominates quantitatively among the cycle acids in a way that differs from tissues of vertebrates.[12] The domination of succinic acid could also be due to some endogenic deviation connected to a citric acid cycle. If the known dehydrogenases in the liver fluke, *e.g.* succinic and malic dehydrogenases,[18,19] are endogenic then at least part of a citric acid cycle could be supposed. Possibly the cycle, if functioning slightly aerobically, could be connected to remnants since the life cycle of *Fasciola hepatica* includes aerobic phases.[20] Anyhow it is known that organisms under anaerobic conditions are able to metabolize citric acid cycle acids in the presence of suitable acceptors.[12] In the absence of O_2, *Escherichia coli* forms succinic acid from fumaric acid [12] and some trypanosomes produce anaerobically citric acid cycle acids from pyruvate.[21] It is also shown that predominantly anaerobic nematodes like *Ascaris* produce succinic acid from fumaric acid.[22] The last mentioned reactions are bound to particles. Possibly the mentioned acids in the mainly anaerobically living *Fasciola hepatica* are in their metabolism bound to particles and connected to the mitochondria poor in inner membranes.

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The Decay of Radiation-Induced Free Radicals in Egg Albumin at Different Water Contents

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Radiation denaturation of proteins in the solid state, measured for example as enzyme inactivation, is being more and more investigated. The water content effect on the radiation damage in seeds [1,2] and starch [2,3] has been well established. The radiation damage in enzymes also seems to be influenced by the water content.[4] For these reasons it was decided to make an investigation of the decay of radiation-induced free radicals in a protein at various water contents and to compare the data with those obtained from studies on whole seeds.[2]

In this investigation egg albumin was used, which was prepared by P. Perlmann according to the method of Kekwick and Cannan.[5,6] The egg albumin, originally containing 19 % water, was equilibrated to different water contents by means of prolonged storage in atmospheres of controlled humidity.[1] The water content was calculated from the loss of weight during desiccation, and was also checked by drying to constant weight at 105°C. The samples were transferred to Pyrex tubes (3 mm inner diameter) and were evacuated three times for 5 min. Between each evacuation, the samples were washed with oxygen-free nitrogen (from British Oxygen) and the tubes were finally filled with nitrogen. The samples were irradiated with ^{60}Co γ-rays during 1 h, and received a dose of 0.43 Mrad. They were measured by electron spin resonance (ESR) within 2 h after irradiation and the decay of radicals was followed an appropriate length of time. Technical data of the ESR equipment and absolute measurements of radicals are described elsewhere.[7] Some samples were opened after the first measurements, and the decay in air was followed. Irradiation and ESR measurements were made at room temperature.

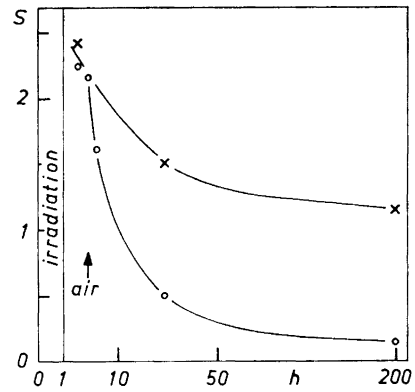

Fig. 1. Decay curves for radicals in egg albumin containing 0.6 % water; upper curve in nitrogen, lower curve in air from the time indicated. S is in units of radicals $\times$ g^{-1} $\times$ 10^{17}.

The ESR spectrum and its change with time agree at least qualitatively with the results of Polatova *et al.*[8] Two typical decay curves are shown in Fig. 1. No simple rate equations can be fitted to the complete curves. The best attempted approximation seems to be a second order reaction if a semi constant level is introduced, as in decay reactions for radicals in seeds.[2]

An extrapolation of the decay curve to time zero, *i.e.* the end of the irradiation period, and a correction of this extrapolated value for the decay during the irradiation, using the previously mentioned second order reaction approximation, gives the total amount of radicals, S_0, which has been induced. This value, of course, does not include such types of radiation-induced radicals which decay so rapidly that they have already vanished completely at the first measurement. In Fig. 2, where S_0 is plotted *versus* water content, it can be seen that there is a strong water content