CHROMATOGRAPHIC DETERMINATION OF PYRUVATE, LACTATE, ACETOACETATE AND ß-HYDROXYBUTYRATE IN FED AND STARVED COD *(GADUS MORHUA).*

By

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ABSTRACT

An ion-exchange HPLC method for the simultaneous determination of pyruvate, lactate, hydroxybutyrate and acetoacetate in organs of cod is described. A feeding experiment with cod was set up to elucidate the impact of dietary fat and carbohydrate and of starvation upon these metabolites. A low and steady blood concentration was measured for pyruvate, ß-hydroxybutyrate and acetoacetate. Blood lactate varied dependent on the dietary carbohydrate level, and disappeared in blood during food deprivation. The level of muscle lactate seemed independent of diet composition and of food deprivation. High levels of ß-hydroxybutyrate were found in the heart of the fish on a high fat, low carbohydrate diet. Acetoacetate was found in the heart from all samplings.

INTRODUCTION

After several studies directed towards the energy utilization in cod (Lie *et al.*, 1986, 1988; Hemre *et al.*, 1989, 1990a,b) there was a need for an analytical method to determine the metabolites ß-hydroxybutyrate and acetoacetate from the anaerobic catabolism of lipids and to determine simultaneously pyruvate and lactate from the anaerobic catabolism of carbohydrate and protein.

Cod seems to utilize efficiently no more than 10% of the dietary fat (on a dry weight basis) as energy when it is fed *ad libitum* on processed feed. Excess is deposited in the liver as triacylglycerides (TAG) and the liver may contain up to 70% of lipids and account for more than 12% of the total fish weight (Lie *et al.*, 1986; 1988). Several studies with carnivorous fish species, including the cod, suggest a suppressed carbohydrate utilization, with diabetic symptoms resembling those of higher animals (Nagai and Ikeda, 1973; Bergot 1979; Hemre *et al.*, 1989, 1990a,b). In rats and mammals this is commonly observed

as depressed glucose oxidation, increased gluconeogenesis, impaired lipogenesis, increased lipid and fatty acid degradation, urinary excretion of glucose, and formation of ketone bodies. Ketone bodies also accumulate during starvation or by feeding a high fat, low carbohydrate diet.

In cod liver glycogen is depleted during starvation (Hemre et al., 1990a), but this is not sufficient to supply the animal with energy when food supply is restricted. The cod survives several weeks of starvation by using primarily muscle protein and to some extent depot-fat to maintain an adequate metabolic pool of carbon substrates (Takama et al., 1985, Hemre et al., 1990a). Anaerobic glycolysis to lactic acid releases only a small fraction of the energy made available when glucose and free amino acids are oxidized completely to carbon dioxide and water. Muscle glycolysis appears to be the predominant anaerobic pathway in rainbow trout (Oncorhynkiss mykiss) and in bullhead catfish brain (Ictalurus rubulosus) (DiAngelo and Heath, 1987). Some fish species are able to increase their anaerobic production of ATP in muscle tissue by the simultaneous catabolism of carbohydrate and amino acids. A variety of end products may result, including succinate, alanine, proprionate, isobutyrate and isovalerate (McDougal et al., 1968).

A method based on ion-exchange high performance liquid chromatography (HPLC) modified from Haller and Lackner (1987) was developed to determine the organic acids pyruvate, lactate, acetoacetate and ß-hydroxybutyrate simultanously. The keto-acids keto-isometylvalerate, keto-isovalerate and keto-isocaproate, derived from the amino acids isoleucine, valine and leucine, respectively, are also measured by this method. A feeding experiment was performed in order to elucidate the energy fluxes in the cod during a feeding experiment with three groups of cod given low fat, medium fat and high fat diets. The fish were fed for 8 weeks followed by 3 weeks of starvation. Pyruvate, lactate, acetoacetate and ß-hydroxybutyrate in blood, heart, liver and fillet were measured by the method described.

MATERIALS AND METHODS

Fish and diets

Three dietary groups with 60 cod of 200 g in each, reared and hatched at the Aquaculture Station Austevoll, were used in this study. The fish were kept in six 1.5 m^3 tanks (replicate groups) and fed ad libitum once a day for 8 weeks. The tanks were supplied with running sea water, temperature and salinity were kept constant at 8°C and 34 gL $^{-1}$, respectively. The fish were fed diets with three fat levels, 3.4%, 5.6% and 12.0% in groups 1, 2 and 3, respectively. Carbohydrate energy (16.8 kJ/g) substituted fat energy (33.6 kJ/g), while

protein energy (17.6 kJ/g) was high (57%) and constant in all three groups. Capelin oil, pre-cooked potato starch and squid mantle were used as the lipid-, carbohydrate- and protein sources in all feeds. The diets contained 1% vitamin and 1% mineral mixtures, for composition see Hemre *et al.* (1991). After 8 weeks of feeding, samples were collected, thereafter 10 fish from each group were starved for 3 weeks before the final sampling. Results from the duplicate tanks were combined as no statistical differences were found between duplicates.

Sample collection

After termination of the feeding and the starving period 5 fish from each tank (10 from each dietary group) were collected and anaesthesized with benzocaine. Blood samples were taken from the caudal vein of anaesthesized fish and collected in tubes containing acid (1.1 M sulphuric acid in 0.05% EDTA), to stabilize the organic acids and to denature the protein. Heart, liver and muscle were immediately frozen on dry ice (-80°C) and kept at this temperature until sample preparation.

Analytical method

Pyruvate, lactate, β-hydroxybutyrate and acetoacetate were separated in a single analytical run on a cation exchange column using dilute sulphuric acid (doubly-distilled water, pH adjusted to 2.00 with sulphuric acid), and monitored by an UV-detector at 208 nm wavelength. Chromatography was performed at 50°C with a Bio-Rad HPX-87H organic acid column (300 x 7.8 mm) protected by a Micro-guard cartridge. This column contains an 8% crosslinked strong cation exhanger of 9μm particle size and is developed for the determination of serum lactate and pyruvate (The Liquid Chromatographer, published by Bio-Rad Laboratories No. 7EG, California, USA, June 1981, pp. 1-8). The pump (Constametric) delivered the effluent at 0.6 ml/min to a detector (Shimadzu, SPD-6AV) connected to a linear recorder (Shimadzu C-R3A chromatopac). Peak heights were measured, and peaks were identified by comparison of retention times with those of standards. Identity was confirmed by «standard addition» to the different organ extracts. All reagents and standards were obtained from Sigma Chemical Co., USA.

Sample preparation

Blood were deproteinized with 1.1 M sulphuric acid, 1:1 v/v, and filtered through a 0.45 μm filter. The organ samples were homogenized and extrac-

ted with 0.1 M H_2SO_4 in 0.05% EDTA (disodium ethylenediaminetetrace-tate). The sample blends were centrifuged at 6000 rpm for 10 min. at 10°C. The supernatant was separated and the sediment was washed twice, recentri-fuged and the washings were combined with the supernatant. The sample preparation was stored at -20°C until HPLC analysis. Prior to injection the supernatants were filtered through a 45 μm filter, no further sample clean up or pH adjustment was found to be necessary. Standards were prepared from stock solutions and filtered.

Statistics

One way ANOVA analysis of variance was used for statistical evaluation of differences between groups. Students t-test was used for comparison of feeding and fasting response within groups.

RESULTS AND DISCUSSION

The analytical method

The resolution and retention time for the compounds depended on several HPLC-conditions, including mobile phase pH and column temperature. The chromatographic conditions described in «material and methods» were pro-ven useful for the determination of pyruvate, lactate, ß-hydroxybutyrate and acetoacetate. This method is also described in the literature for the determina-tion of small molecular weight organic acids and of taurine in fish (Haller and Lackner, 1987). The column used, Aminex HPX-87H, a strong cation-exchange column material, separates alcohols and carbohydrates as well as organic acids. The organic acids are detected, whereas most sugars and alcohols are not detected at the wavelength used unless they are present in very high concentrations (Haas *et al.*, 1988). The separation of a standard mixture is shown in Figure 1. Table 1 shows retention times and detection limits of pyruvate, lactate, ß-hydroxybutyrate, acetoacetate and retention times of some other organic acids that might interfere with one or more of the analysed acids. Sulphuric acid (0.1 M) containing 0.05% EDTA was used for extraction of the organic acids from organs as interferring absorbing peaks at the detection wavelenght were present when perchloric acid or trichloracetic acid extraction was used as also found by Haas *et al.* (1988). EDTA was chosen as a good chelating agent to mask any divalent metal ions present in the sample preparations (Ashoor and Welty, 1984). The EDTA has a shorter retention time than those of the organic acids of interest, and did not interfere with the quantitative determination of the acids (Figure 1 and Table 1). Our

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Table 1: Retention times of the investigated acids and other possible interferring acids (all at pH = 2.00, flowrate 0.6 ml/min and column temperature 50 °C).

Organic acid	Retention time
EDTA	5.41
Pyruvate	8.83[1]
Ketoisovalerate	9.19
Succinate	10.51
Lactate	11.37[2]
Metylvalerate	11.90
ß-hydroxybutyrate	12.30[3]
Acetoacetate	12.81[4]
Ketoisocaproate	14.22
Propionate	17.23

[1] Detection limit (20µl injection volume) $8.7 * 10^{-3}$ mmol/l
[2] Detection limit (20µl injection volume) $8.9 * 10^{-4}$ mmol/l
[3] Detection limit (20µl injection volume) 0.010 mmol/L
[4] Detection limit (20µl injection volume) 0.010 mmol/L

Figure 1. Separation of lactate, pyruvate, -hydroxybutyrate and acetoacetate by HPLC. RT = 5.407; EDTA, RT = 8.825; pyruvate, RT = 11.368; lactate, RT = 12.303; ß-hydroxybutyrate and RT = 12.812; acetoacetate.
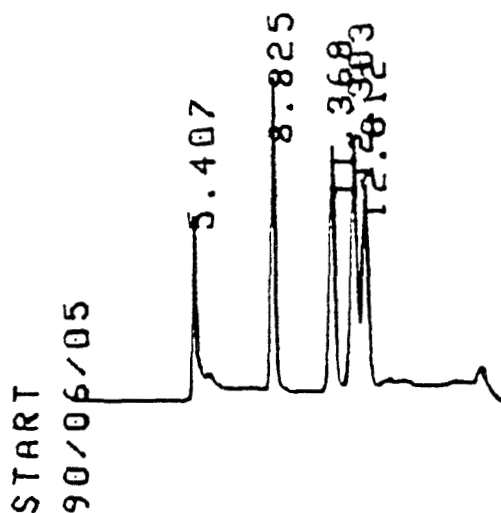
unidentified peaks are probably short chain fatty acids or taurine (Haller and Lackner, 1987) and were not identified as ketoisovalerate, ketoiso-metylvalerate or ketoisocaproate (Figure 2).

Blood lactate values measured after the feeding and starvation periods are shown in Table 2. A significant higher blood lactate level ($p < 0.05$) was found in group 1 compared to groups 2 and 3 after the feeding period. We have earlier suggested that blood lactate origins both from free amino acids and from glucose (Hemre *et al.*, 1990; 1991). In this experiment, however, all groups were fed diets with equal amounts of protein and the higher lactate levels in group 1 may originate from the higher carbohydrate intake in this group. In all groups a significant reduction in the lactate level ($p < 0.01$) was found after 3 weeks of fasting. The levels of pyruvate (0.2-0.4 mmol/l) were independent of dietary fat and carbohydrate content and of food deprivation.

Figure 2. The separation of organic acids in cod liver. No peaks were identified as pyruvate, lactate, hydroxybutyrate, acetoacetate, ketoisovalerate, succinate, metylvalerate, ketoisocaproate or propionate.
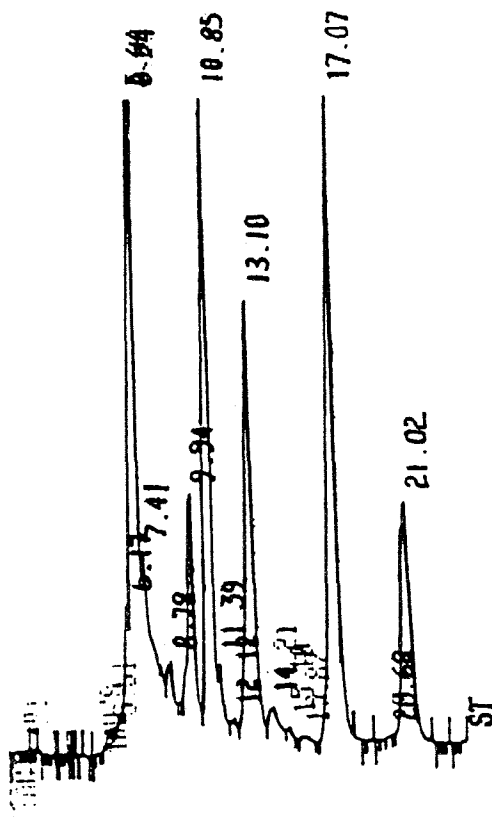
Table 2: Pyruvate (mmol/l), lactate (mmol/l), ß-OH-butyrate (mmol/l) and acetoacetate (mmol/l) in blood plasma from cod fed different fat and carbohydrate levels (group 1, 2 and 3 = low fat, medium fat and high fat, respectively) and thereafter starved for 3 weeks.

	Group 1	Group 2	Group 3
Lactate F[1]	2.9 (1.3)*	1.9 (0.6)	1.9 (0.7)
Lactate S[2]	0.6 (0.3)	0.3 (0.1)	0.4 (0.1)
Pyruvate F	0.3 (0.1)	0.3 (0.1)	0.2 (0.1)
Pyruvate S	0.4 (0.3)	0.4 (0.4)	0.4 (0.3)
ß-OH-butyrate FS[4]............................	trace[3]	trace	trace
Acetoacetate FS	trace	trace	trace

* Numbers in paranthesis are standard deviation values, each number represents analysis from 10 fish.
[1] F = fed fish (8 weeks adaptation to the different diets)
[2] S = starved fish (3 weeks starvation)
[3] trace = small amounts, limits the detections levels of the method.
[4] FS = results from both fed and starved fish

Trace amounts of ß-hydroxybutyrate and acetoacetate were found in the blood samples. These results are in accordance with findings from fed and starved bass *(Dicentrarcus labrax)* (Zammit and Newsholm, 1979). Variations due to dietary lipid intakes or 3 weeks of starvation, were not demonstrated. Little information is available regarding ketone bodies and their role in fish. However, according to Sargent *et al.* (1989) ketone body production does not occur in teleost fish subjected to starvation. A constant blood concentration is thus reported for several species (DiAngelo and Heath, 1987; Thillart *et al.*, 1982; Jonas and Bilinsky, 1965) and further increased levels of ß-hydroxybutyrate and acetoacetate in fish organs has been measured under special conditions like anoxia (DiAngelo and Heath, 1987) and spawning (Jonas and Bilinsky, 1965). Also detection of higher mucus levels of ß-hydroxybutyrate in mullet *(Mugil cephalus)* subjected to starvation was found by Ramos and Smith (1978).

Table 3 shows the variation in the measured organic acids in muscle tissue. Unlike the levels found in blood, no significant differences were found in muscle lactate after the feeding period. The source of muscle lactate may be catabolized amino acids and/or glycogen (Driedzig and Hochachka, 1976). As the dietary protein levels were equal in all three groups, this suggests that

Table 3: Pyruvate (mmol/kg), lactate (mmol/kg), ß-OH-butyrate (mmol/kg) and acetoace-
tate (mmol/kg) in muscle from cod fed different fat and carbohydrate levels (group
1, 2 and 3 = low fat, medium fat and high fat, respectively) and thereafter starved
for 3 weeks.

	Group 1	Group 2	Group 3
Lactate F[1]	1.2 (0.6)*	1.2 (0.6)	1.6 (1.1)
Lactate S[2]	0.7 (0.3)	0.7 (0.2)	0.6 (0.3)
Pyruvate F	trace[3]	trace	trace
ß-OH-butyrate FS[4]	nd[5]	nd	nd
Acetoacetate FS	nd	nd	nd

* Numbers in paranthesis are standard deviation values, each number represents analysis
  from 10 fish.
[1] F = fed fish (8 weeks adaptation to the different diets)
[2] S = starved fish (3 weeks starvation)
[3] trace = small amounts, limits the detections levels of the method.
[4] FS = results from both fed and starved fish
[5] nd = below detection level of the method

lactate in muscle does not vary with the dietary carbohydrate content in this
strict carnivorous fish species, in accordance with earlier suggestions (Hemre
et al., 1991). The pyruvate levels in muscle were below the detection limit of
the method and therefore no variation could be found. Acetoacetate and
ß-hydroxybutyrate were not detected in the muscle extracts. These results
indicate that fat is not a major energy substrate in the muscle cells in cod,
either at good food supply or at food deprivation, as also suggested by Lie *et al.*
(1986, 1988).

In heart even lower lactate concentrations than in muscle were found after
feeding, independent of the dietary composition. After 3 weeks of starvation
no lactate was detected. Pyruvate was not detected in the heart of fed or
starved cod. The heart was the only organ responding to a high fat, low
carbohydrate diet showing high levels of ß-hydroxybutyrate in group 3 after
feeding. This is similar to observations on rats and mammals. After 3 weeks of
starvation a lower level of ß-hydroxybutyrate was seen in group 3, and a
similar level was also seen in group 2. In mammals, ketone bodies transfer
acetyl groups, derived from lipids, from the liver to other tissues (Hawkins,
1985). ß-hydroxybutyrate is converted to acetoacetate which is then conver-
ted to its CoA derivative for the use in the TCA cycle. ß-hydroxybutyrate is
the precursor of acetoacetate, which was measured at an almost steady state

Table 4: Pyruvate (mmol/kg), lactate (mmol/kg), ß-OH-butyrate (mmol/kg) and acetoacetate (mmol/kg) in heart from cod fed different fat and carbohydrate levels (group 1, 2 and 3 = low fat, medium fat and high fat, respectively) and thereafter starved for 3 weeks.

	Group 1	Group 2	Group 3
Lactate F[1]	0.8 (0.2)*	0.9 (0.3)	1.0 (0.4)
Lactate S[2]	nd[5]	nd	nd
Pyruvate FS[4]	nd	nd	nd
ß-OH-butyrate F	nd	nd	6.4 (2.3)
ß-OH-butyrate S	nd	0.9 (0.2)	1.1 (0.5)
Acetoacetate F	1.1 (0.3)	1.0 (0.5)	1.1 (0.2)
Acetoacetate S	1.4 (0.7)	0.8 (0.3)	0.9 (0.6)

* Numbers in paranthesis are standard deviation values, each number represents analysis from 10 fish.
[1] F = fed fish (8 weeks adaptation to the different diets)
[2] S = starved fish (3 weeks starvation)
[4] FS = results from both fed and starved fish
[5] nd = below detection level of the method

in the heart. Evidently the cod heart utilized small amounts of ketone bodies in all dietary groups, with a steady food supply as well as during periods of food deprivation. Enzyme activities of 3-oxoacid CoA thiolase, a key enzyme in ketone body utilization, was found in the heart of plaice *(Pleuronectes platessa)*, bass and mackerel *(scombrus scombrus)* (Zammit and Newsholme, 1979), suggesting the use of ketone bodies as energy sources. According to Zammit and Newsholme (1979), Zammit *et al.* (1979) and Henderson *et al.* (1984) 3-hydroxybutyrate dehydrogenase, a key enzyme in ketone body production in mammals, is lacking in the livers of teleost fish. However, Phillips and Hird (1977) and Zammit *et al.* (1979) suggested that 3-oxo acid CoA transferase, found in livers of teleost fish, catalysed the conversion of acetyl CoA into acetoacetate for the formation of ketone bodies. In the carnivorous bullfrog *(Rana catesbeiane)* DeRoos and Rumpf (1987) measured a steady blood flow of ketone bodies. Its natural diet composition (high protein, low carbohydrate) and eating habits are similar to those of the cod. Cowey *et al.* (1981), Rumsey (1981) and Hughes *et al.* (1983) measured high catabolic activity in a number of enzymes involved in protein catabolism in the rainbow trout, suggesting these enzymes to be «permanently set» to handle high protein diets.

Conclusions

There seems to be a low and constant level of ketone bodies and pyruvate in the blood of the cod, independent of dietary fat and carbohydrate as well as of starvation. The constant pyruvate levels in organs, suggests the catabolism of amino acids and glucose in this experiment to be constant. The only organ utilizing ketone bodies at measureable amounts was the heart, with dietary dependence of ß-hydroxybutyrate level, but no differences between organs were found for acetoacetate.

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