NAD- AND NADP-MALIC ENZYMES IN SPERMATOZOA OF MAMMALS AND FISH

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1. Introduction

Previous work from this laboratory indicated that sperm of salmon or cod when incubated with pyruvate, labelled with ^{14}C in any position, yielded radioactive oxaloacetate, malate, aspartate and α-ketoglutarate [1]. These results left little doubt as to the ability of fish sperm to fix carbon dioxide and this was unequivocally documented when it was found that cod sperm incubated in the presence of $^{14}CO_2$ incorporated label into keto-, amino and other organic acids, lipids, proteins, and nucleic acids [2,3]. The discovery of a carbon dioxide fixation by fish sperm was confirmed in sperm of other species such as, ram [4] and bull [5]. In the course of our search for enzymes involved in the fixation of carbon dioxide in the testes of rabbit and fish, we were able to demonstrate the occurrence of two malic enzymes (EC 1.1.1.39 NAD-malic dehydrogenase, decarboxylating; and EC 1.1.1. 40 NADP-malic dehydrogenase decarboxylating) requiring NAD^+ and $NADP^+$ respectively [6–8]. These latter findings prompted us to look for a similar situation in sperm of mammals and fish. It has become evident from the present work that although spermatozoa exhibited demonstrable species differences in the relative activity of malic enzymes, they all possessed two malic enzymes, one of them was active with NAD^+ or $NADP^+$.

2. Materials and methods

Fresh sperm from man, bull, salmon and cod were washed twice with saline to which penicillin was added. Washed sperm were then suspended in a cold medium that contained sucrose (0.25 M), Tris (0.05 M), reduced glutathione (0.1%, w/v), EDTA (0.1%, w/v), and penicillin (10 000 i.u./ml), pH 7.00. Suspensions of sperm were immediately stored frozen at −40°C until they were ready for use. For malic enzyme assays, frozen sperm were sonicated until they have completely thawed, placed in an ice bath for 5 min, and then sonicated again for a total of 2 min interrupted for a 3 min cooling period every 30 sec. The sonicated sperm were centrifuged at 27 000 *g* in the cold for 15 min, and the supernatant fluid was separated for use in incubations. Malic enzyme activity was determined as $^{14}CO_2$ produced from the decarboxylation of [^{14}C]4-malate. The assay was carried out in a Warburg flask with two side-arms at 25°C. Unless otherwise mentioned, the incubation mixture contained (μ moles in 2.00 ml): [^{14}C]4-malate, 50 (total activity: 2 μC); $MgCl_2$ (or $MnCl_2$) 10; NAD^+ (or $NADP^+$) 3; Tris, 300; and a suitable aliquot of the fraction being examined; the final pH was 7.00. [^{14}C]4-malate was obtained from Calatomic, Los Angeles, California, and was purified by using two-way ascending paper (Whatman No. 1) chromatography [9] and the pure radioactive malate was detected using Kodak no-screen film, and then eluted. An equilibrium period of 5–7 min was allowed and the reaction started by adding the [^{14}C]4-malate from one of the side-arms into the central chamber. The incubation lasted for 5 min, except in experiments dealing with the effect of incubation period on enzyme activity. In all cases the reaction was terminated by tipping 0.2 ml of 3 N perchloric acid to the incubation mixture. $^{14}CO_2$ evolving during the incubation period was trapped by a strip of filter paper saturated with 0.3 ml of hyamine hydroxide 10-X. In all experiments a blank treatment was used to correct for any non-enzymatic decarboxyla-

tion of [^{14}C]4-malate, and it was evident that the counts for radioactivity from such treatments were extremely low. In all cases it was found that the decarboxylation of malate was dependent on the presence of malate, nucleotide (NAD^+, or $NADP^+$), and Mg^{2+} or Mn^{2+}. In a few experiments pyruvate produced at the end of the incubation period was determined [10]. Protein concentration, in supernates of sonicated sperm, was determined according to Lowry, Rosebrough, Farr and Randall [11].

Vertical starch gel electrophoresis was carried out using a Buchler apparatus. Starch gel was prepared as 13% starch in the same medium used for suspending the washed sperm (i.e. sucrose-EDTA-reduced glutathione–Tris). The bridge buffer was composed of: reduced glutathione (0.1%, w/v), sucrose (0.25 M), and potassium phosphate (0.1 M for the cathode and 0.0167 M for the anode), pH 7.00. The use of this discontinuous buffer system proved to be useful in maintaining the pH for at least 24 hr. The gel electrophoresis was carried out in the cold (4°C) at 50 V for 20 hr at the end of which strips of gel were incubated in a staining mixture containing: L-malate (50 mM), $MgCl_2$ or $MnCl_2$ (10 mM), Tris (300 mM), NAD^+ or $NADP^+$ (3 mM), nitro blue tetrazolium (2 mM), phenazine methosulphate (0.2 mM), and cysteine sulfinate (5 mM), to transaminate with and thus remove any oxaloacetate formed, pH 7.00.

Table 1
Malic enzyme activity in supernates of sonicated spermatozoa of mammals and fish*

Sperm of:	Co-factor	With Mg^{2+}	With Mn^{2+}
Man	NAD^+	2.0 ± 0.11	7.9 ± 0.12
	$NADP^+$	2.5 ± 0.13	5.0 ± 0.24
	$NAD^+ + NADP^+$	3.8 ± 0.22	10.9 ± 0.45
Bull	NAD^+	3.4 ± 0.14	13.8 ± 0.54
	$NADP^+$	6.6 ± 0.26	9.5 ± 0.36
	$NAD^+ + NADP^+$	9.8 ± 0.54	22.1 ± 1.00
Salmon	NAD^+	37.5 ± 1.61	188 ± 5.71
	$NADP^+$	272 ± 7.83	334 ± 8.63
	$NAD^+ + NADP^+$	288 ± 6.94	473 ± 9.14
Cod	NAD^+	43.0 ± 2.14	154 ± 3.66
	$NADP^+$	300 ± 5.97	311 ± 6.82
	$NAD^+ + NADP^+$	309 ± 6.88	391 ± 9.45

*Malic enzyme activity is determined as $^{14}CO_2$ produced from the decarboxylation of [^{14}C]4-malate (nmole $^{14}CO_2$/mg of protein/5 min). Mean values of 6 experiments ± standard error.

Table 2
The effect of two levels of NAD^+ or $NADP^+$ on malic enzyme activity in supernates of sonicated spermatozoa of mammals and fish*

Sperm of:	Co-factor	Amount of nucleotide/incubation: 3 μmoles	6 μmoles
Man	NAD^+	7.8	7.9
	$NADP^+$	5.1	4.9
Bull	NAD^+	13.9	13.5
	$NADP^+$	9.4	9.4
Salmon	NAD^+	185	188
	$NADP^+$	339	334
Cod	NAD^+	158	156
	$NADP^+$	310	314

*Malic enzyme activity is determined as $^{14}CO_2$ produced from the decarboxylation of [^{14}C]4-malate (nmole $^{14}CO_2$/mg of protein/5 min). Values are averages of 3 experiments. All incubations were carried out in the presence of Mn^{2+}.

3. Results

Malic enzyme activity, as indicated by the decarboxylation of [^{14}C]4-malate, in the supernates of sonicated sperm, was dependent on the presence of NAD^+ or $NADP^+$, but more activity was observed in the presence of both nucleotides than when one of them was used (table 1). In sperm of both man and bull, the relative activity of malic enzyme(s) was greater with Mn^2 than with Mg^{2+}, and this was particularly the case in treatments with NAD^+ (approx. 4 times); also in the presence of Mn^{2+} the decarboxylation of malate was higher with NAD^+ than with $NADP^+$, whereas the reverse was true in the presence of Mg^{2+} (table 1). The decarboxylation of malate by supernates of sonicated salmon and cod sperm was substantially higher than that by those of mammalian sperm (more than 10 times with NAD^+, and more than 30 times with $NADP^+$, table 1). In both salmon and cod, malic enzyme activity was greater with $NADP^+$ than with NAD^+; in treatments with NAD^+ the decarboxylation of malate was higher with Mn^{2+} than with Mg^{2+} (approx.

5 and 3.5 times in salmon and cod respectively, table 1).

Additional experiments were carried out to examine whether the amount of NAD^+ or $NADP^+$ used in incubations reported in table 1, namely 3 μmoles/flask, was sufficiently saturating. In these experiments Mn^{2+} was the heavy metal of choice, and comparison was made between incubations that received 3 μmoles of nucleotide/flask and those that had 6 μmoles/flask; it was evident that doubling the amount of NAD^+, or $NADP^+$ did not promote the oxidative decarboxylation of malate by sperm sonicates (table 2). Furthermore, increasing the concentration of malate, or heavy metal (Mn^{2+}, or Mg^{2+}) beyond that used in the present investigation caused no significant increase in the $^{14}CO_2$ evolved. These findings led to the conclusion that under the present experimental conditions at pH 7.00, the concentrations of all substrates and co-factors were saturating in all cases examined.

The existence of two malic enzymes in sperm of each of the four species studied was further supported by the finding that the decarboxylation of malate was linear with time for at least 10 min (figs. 1 and 2) and also the production of pyruvate was stoichiometrical with that of $^{14}CO_2$ evolved from the decarboxylation of [^{14}C]4-malate in incubations that lasted 5 min

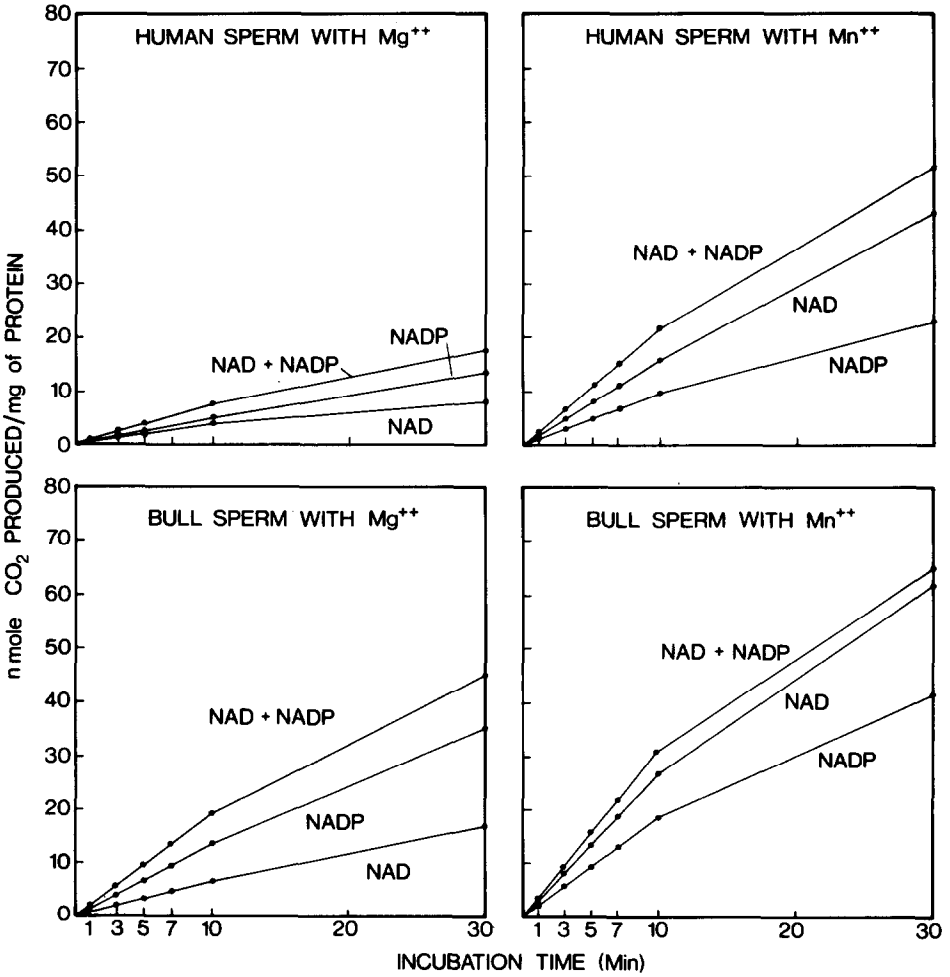

Fig. 1. The relationship between the oxidative decarboxylation of malate and length of incubation period by supernates of sonicated sperm of mammals.

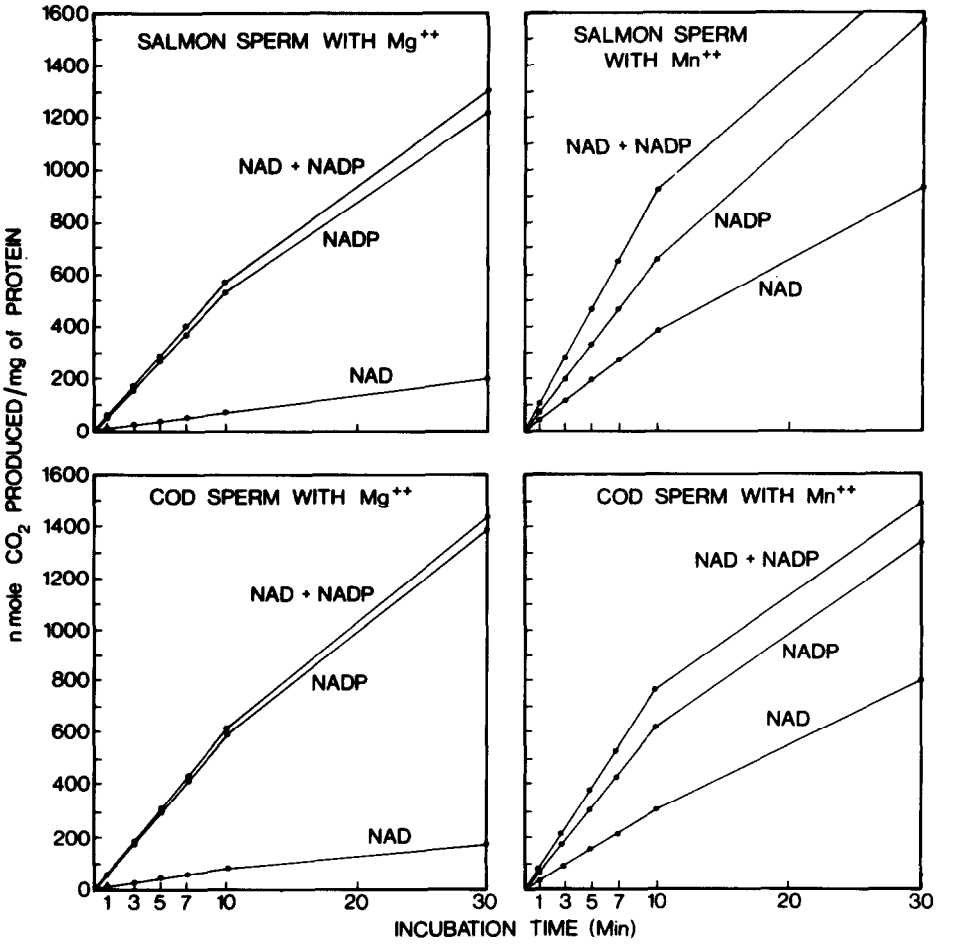

Fig. 2. The relationship between the oxidative decarboxylation of malate and length of incubation period by supernates of sonicated sperm of fish.

(table 3). However, it must be mentioned that when the incubation period was extended beyond 5 min, the production of pyruvate was less than that of $^{14}CO_2$, but more lactate was produced; a possible explanation is that the concentration of pyruvate produced during the 5-min incubation period was less than that of the K_m value of lactate dehydrogenase.

Vertical starch gel electrophoresis experiments demonstrated that mammalian sperm possessed at least two malic enzymes: one is NAD-specific and the other is active with NAD^+ or $NADP^+$. In fish sperm, however, cod showed two malic enzymes that reacted with NAD^+ or $NADP^+$, whereas salmon had one NADP-specific enzyme and another that was active with NAD^+ or $NADP^+$.

4. Discussion

After the demonstration of the presence of two malic enzymes requiring NAD^+ and $NADP^+$ respectively in one compartment (mitochondrion or cytosol) of an animal tissue, namely rabbit and cod testis [6–8] other workers verified the existence of these two enzymes in other animal tissues, e.g. mitochondria and cytosol of rabbit and cod ovaries [12], mitochondria

Table 3
Production of pyruvate and $^{14}CO_2$ from [^{14}C]4-malate by supernates of sonicated spermatozoa of mammals and fish*

Sperm of:	Co-factor	Pyruvate produced	$^{14}CO_2$ Produced
Man	NAD^+	8.00	7.9
	$NADP^+$	5.1	5.1
Bull	NAD^+	13.7	14.0
	$NADP^+$	9.7	9.9
Salmon	NAD^+	186	188
	$NADP^+$	338	340
Cod l	NAD^+	156	155
	$NADP^+$	308	310

*nMole pyruvate (or $^{14}CO_2$) produced/mg protein/5 min. Values are averages of two experiments. All incubations were carried out in the presence of Mn^{2+}.

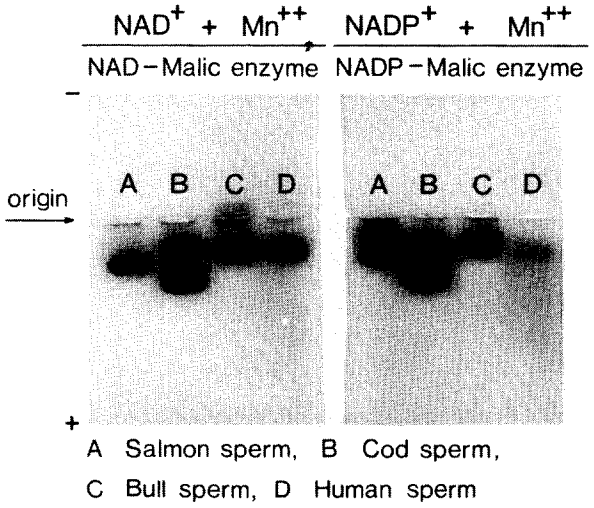

Fig. 3. Starch gel electrophoresis of NAD- and NADP-malic enzymes in supernates of sonicated sperm of mammals and fish.

of rabbit heart [13,14], cod eggs [15], and mitochondria of rat adrenal cortex [16]. To the best of our knowledge, the present investigation presents the first proof of the occurrence of two malic enzymes in sperm of mammals and fish. That mammalian sperm contained a NAD-specific malic enzyme could offer an explanation to the finding that NADH promoted CO_2 uptake by bull sperm [17].

It is interesting to note that although spermatozoa exhibited demonstrable species differences in the relative activity of malic enzymes, they all possessed two malic enzymes, one of them was active with NAD^+ or $NADP^+$. It is also noteworthy to refer to the observation that the mitochondria of rat adrenal contained two malic enzymes, one is NADP-specific and unaffected by succinate or ATP, whereas the other is active with NAD^+ or $NADP^+$ and allosterically activated by succinate, and inhibited by ATP [16]. The existence of two malic enzymes in sperm may play an important regulatory role in the replenishing of C-4 dicarboxylic acids, through carboxylation of pyruvate to malate, and also in the generation of NADPH. The distribution of these enzymes in the mitochondria and cytosol of sperm warrants further investigation, particularly in view of the finding that all the malic enzymes reported in the present study showed relatively higher activity with Mn^{2+} than with Mg^{2+}.

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