

## Pyridine Nucleosidase in Bull Semen

### II.\* Biochemical Properties

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#### *Abstract*

It is most likely a single enzyme (NAD<sup>+</sup> nucleosidase) present in semen from most bulls which hydrolyses the ribosyl pyridinium bond in both NAD and NADP. This conclusion is based on the following results: (i) each of 12 semen samples containing nucleosidase activity hydrolysed NAD at the same rate as NADP ( $r = 0.99$ ); (ii) other untreated semen samples from different bulls which did not hydrolyse NAD were also inactive against NADP; (iii) enzyme denaturation produced by preliminary heating of semen filtrates for 15 min at varied temperatures or by heating at 55°C for varied time intervals caused similar reductions in the rates of NAD and NADP hydrolysis; and (iv) nicotinamide inhibited enzyme activity to the same degree using either NAD or NADP as the substrate.

#### **Introduction**

The biological significance of a soluble pyridine nucleosidase in bull semen originally identified by Leone and Bonaduce (1959) has not been elucidated. Bistocchi *et al.* (1968) showed that ejaculated bull spermatazoa possessed less than 35% of the levels of NAD and NADP found in epididymal spermatazoa. It has not been resolved whether NAD and NADP are hydrolysed by the same enzyme which cleaves the ribosyl pyridinium bond. Macmillan *et al.* (1975) found that the concentration of seminal pyridine nucleosidase varied between bulls from zero to 1470 enzyme units/ml semen but only NAD was used as the enzyme substrate. This wide range in enzyme concentration facilitated studies to determine whether more than one pyridine nucleosidase was involved.

#### **Materials and Methods**

The procedures used for semen sampling and storage, and for assaying nucleosidase activity have been reported previously (Macmillan *et al.* 1975).

The effect of temperature on enzyme inactivation was assessed by heating 1 ml of the diluted semen filtrate for 15 min at 40, 45, 50, 55, 60 or 70°C followed by rapid cooling to 20°C. The rate of enzyme inactivation was measured by heating similar semen samples at 55°C for from 5 to 40 min.

The effect on nicotinamide inhibition on enzyme activity was measured by adding from 0.39 to 100 mmol of nicotinamide in 0.05 ml volume to 1.0 ml of the semen filtrate prior to the addition of NAD or NADP. Standards for each concentration of nicotinamide to which neither NAD nor NADP was added were also incubated.

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## Results

A diluted semen sample from each of four bulls with varied enzyme concentrations was assayed in duplicate using 1.0, 0.5, 0.25 or 0.1 ml of the semen filtrates and NAD or NADP as substrate. The correlation coefficient of enzyme activity expressed as units per incubated sample for NAD and NADP was 0.99 ( $P < 0.001$ ) (Fig. 1).

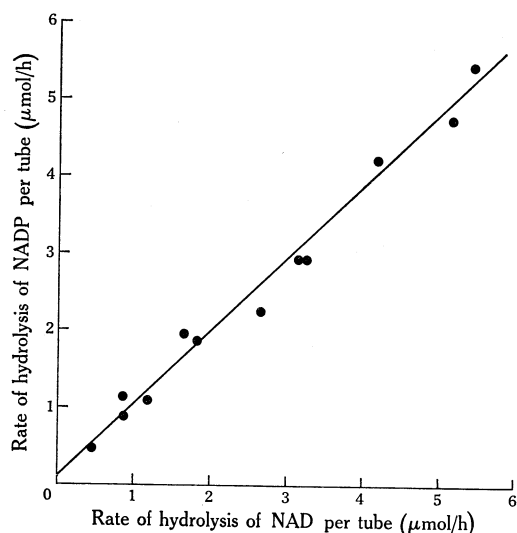


Fig. 1. Rates of hydrolysis of NAD and NADP at 36°C with varied amounts of diluted semen filtrates (see text).  
 $\hat{y} = 0.94x + 0.12$  ( $r = 0.99$ ).

Semen samples from each of eight previously identified bulls which did not hydrolyse NAD were also inactive when NADP was used as substrate.

When two semen samples with high enzyme concentrations (600 and 480 units/ml semen) produced by one bull and a single sample from a second bull with a lower enzyme concentration (200 units/ml) were heated for 15 min at selected temperatures from 40 to 70°C, the subsequently assayed levels of enzyme activity using NAD and NADP differed between samples but were similar within each semen sample (Fig. 2). Enzyme activity was reduced following heating at 55°C and the degree of inactivation increased with higher temperatures.

Table 1. Effect of nicotinamide inhibition on the rates of hydrolysis of NAD and NADP

Substrate activity measured as  $\mu$ mole substrate hydrolysed per hour, mean of two assays

Nicotinamide content (mmol)	Substrate		Nicotinamide content (mmol)	Substrate	
	NAD	NADP		NAD	NADP
0	3.15	3.00	6.25	1.73	2.17
0.39	2.70	3.07	25	1.20	1.35
1.56	2.48	2.77	100	0.52	0.60

Diluted semen samples from these two bulls were also heated for up to 30 min at 55°C. The logarithm of enzyme activity declined at a linear rate with respect to time

(Fig. 3). Regression analyses showed that these decreases were significantly linear ( $P < 0.001$ ) but the slopes obtained from using NAD or NADP as the substrate with each semen sample were similar ( $P > 0.10$ ).

A duplicated assay to determine the inhibitory action of nicotinamide on enzyme activity showed that the degree of inhibition was related to nicotinamide concentration. The effect was similar irrespective of whether NAD or NADP was used as substrate (Table 1).

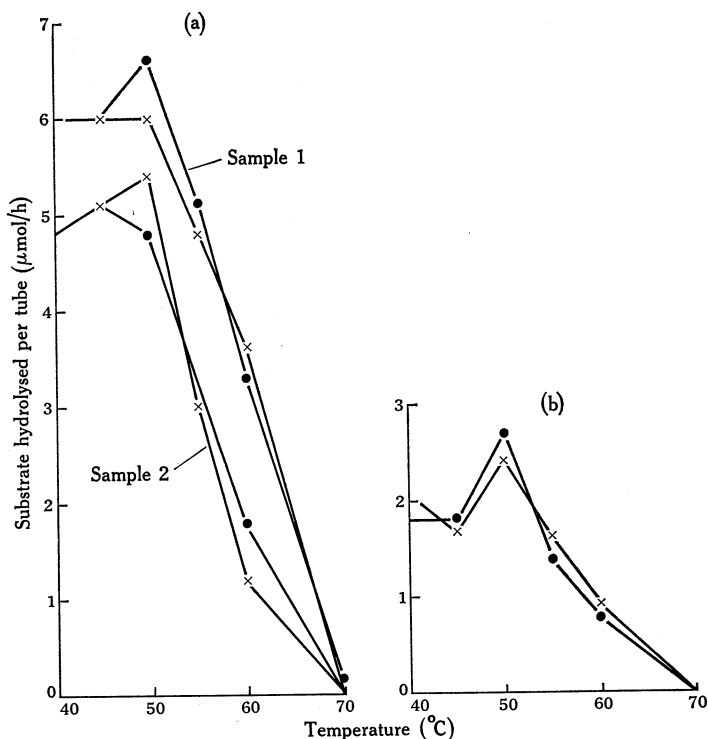


Fig. 2. Effects of heating diluted semen filtrates for 15 min at selected temperatures on pyridine nucleosidase activity with NAD (x) and NADP (●) as substrates. (a) Bull A, (b) bull B.

## Discussion

In Part I of this series (Macmillan *et al.* 1975) identifying a soluble pyridine nucleosidase in bull semen it was suggested that there were two distinct enzymes, one which hydrolysed NAD and a second specific for NADP (Leone and Bonaduce 1959). Improved methods of enzyme purification used by Abdel-Latif and Alivisatos (1962) did not resolve the question as the ratio of activity using NAD as substrate compared to that using NADP varied with increasing purification and with storage. Yuan and Anderson (1971) subsequently produced a highly purified enzyme identified as NAD<sup>+</sup> nucleosidase (EC 3.2.2.5). This single enzyme had a molecular weight of 36 300 (Yuan *et al.* 1972) and had similar  $K_m$  values with NAD and NADP (Yuan and Anderson 1973). This did not eliminate the possibility that another enzyme which preferentially hydrolysed NADP may not have been removed during purification.

It seemed important to clarify this point as the presence of significant quantities of more than one pyridine nucleosidase could influence the interpretation of results obtained by Macmillan *et al.* (1975) relative to the mode of inheritance of enzyme concentration.

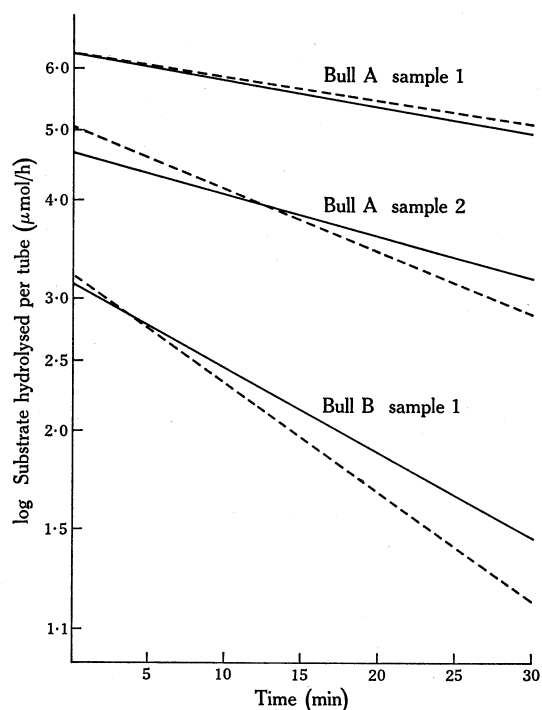


Fig. 3. Effects of heating diluted semen filtrates at 55°C for selected times on pyridine nucleosidase activity with NAD (—) and NADP (---) as substrates.

Semen samples from seven selected sires of three recognized dairy breeds which did not hydrolyse NAD were also inactive with NADP. This absence of activity was not due to enzyme degradation during storage, or to the presence of an inhibitor in semen from these bulls (Macmillan *et al.* 1975). When semen samples containing enzyme activity were assayed, their activity in terms of the rate of hydrolysis of NAD was similar to the rate of hydrolysis of NADP (Fig. 1). The effects of enzyme denaturation either by varying temperature (Fig. 2), or by varying time at a constant temperature of 55°C (Fig. 3) were similar relative to the rates of hydrolysing NAD and NADP. Abdel-Latif and Alivisatos (1962) reported that nicotinamide could completely protect NAD from hydrolysis. While this inhibitory effect with NAD as substrate has been confirmed and the nature of the inhibition defined (Yuan and Anderson 1972), our results show that nicotinamide is equally effective in inhibiting the hydrolysis of NADP (Table 1).

These results which show that NADP is hydrolysed at the same rate as NAD following a variety of treatments involving enzyme inactivation or inhibition suggest that only a single soluble pyridine nucleosidase present in semen from most bulls hydrolyses the ribosyl pyridinium bond.

In contrast to results obtained by Abdel-Latif and Alivisatos (1962), we found that 100 mmol nicotinamide/ml did not completely inhibit enzyme activity. The degree of activity which remained was similar to that recorded by Wheat *et al.* (1960) who used a

similar concentration of nicotinamide. These differences may be due to the composition of the assay media.

Bull semen contains other enzymes which may destroy NAD or NADP. These include nucleotide pyrophosphatase (EC 3.6.1.9) and 5'-nucleotidase (EC 3.1.3.5). High concentrations of the latter enzyme have been measured (Heppel and Hilmoe 1951) whereas nucleotide pyrophosphatase activity is comparatively minor (Wheat *et al.* 1960). NAD is not an ideal substrate for either of these enzymes and both require magnesium ions (5–8 mmol/ml) (Heppel and Hilmoe 1951; Brownlee and Wheat 1960). The average concentration of magnesium in bull semen is only 5  $\mu$ mol/ml (Mann 1964). As the greatest amount of semen used in our assays was equivalent to 0.01 ml, the activity of these two enzymes should have been minimal. Either the samples which did not contain seminal nucleosidase also did not contain other enzymes which destroy nucleotides, or the presence of these other enzymes did not influence results under our assay conditions.

## References

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