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Heat Lability of NADPH at Physiologic *p*H

S. Robert Young[1] and Arthur D. Bloom[1]

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Recently, the procedure of induced fluorescence (Lowry *et al.*, 1957) was adapted for the microassay of glucose 6-phosphate dehydrogenase (G6PD) in preimplantation mammalian embryos (Brinster, 1970). In this assay, samples were incubated in the presence of NADP, glucose 6-phosphate, $MgCl_2$, EDTA, and crystalline bovine serum albumin in tris–HCl buffer (*p*H 7.5) for periods of 30 and 60 min, and the enzymatic reaction was terminated by immersing the sample tubes in boiling water for 2 min. Succeeding steps involved destruction of the excess NADP with dilute NaOH (Lowry *et al.*, 1957) and the production of a fluorescent product by a mixture of strong base and hydrogen peroxide.

In the course of our investigation of G6PD activities in preimplanted rabbit embryos, inconsistent results obtained by using the above method led us to examine the individual steps of the procedure. It is well known that NADPH is stable at alkaline *p*H (Lowry *et al.*, 1961). We report here our findings on the temperature lability of NADPH at physiological *p*H.

Solutions of NADPH (Sigma Chemical Company) were prepared in a concentration range suitable for direct fluorometric analysis (1×10^{-6} to 9×10^{-6} M) using 0.05 M tris–HCl buffer at *p*H 7.5. Aliquots of these solutions were placed in a water bath at 22, 60, or 100 C, and after 0.5, 1, 2, 4, 8, and 16 min the tubes were transferred to an ice bath. After warming to room temperature, the native fluorescence of NADPH was recorded in an Aminco-Bowman spectrophotofluorometer (SPF).

In separate experiments, the complete enzyme assay was done using

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[1] Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan.

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Table I. Effect of 0.15 N NaOH on G6PD

Minutes incubation at 37 C	Fluorometric units: 5 μl control enzyme[a] per 1 ml reaction mixture[c]	Fluorometric units: 5 μl treated enzyme[b] per 1 ml reaction mixture[c]
0	0.40	0.38
30	3.20	0.50
60	5.30	0.66

[a] Equal amounts of stock yeast G6PD (0.1 IU/ml) and tris buffer[c] mixed in ice bath for 1 min.
[b] Equal amounts of stock yeast G6PD and 0.15 N NaOH mixed in ice bath for 1 min.
[c] Tris buffer (*p*H 7.8), NADP (0.1 mM), glucose 6-phosphate (1.0 mM), $MgCl_2$ (10 mM), EDTA (1.0 mM), and crystalline bovine serum albumin (0.05%).

yeast G6PD (Sigma). The reaction mixture of Brinster (1970) was used except that the NADP concentration was reduced tenfold to lower the blank values. The samples were incubated for 60 min at 37 C, and the reaction was stopped either by immersing the tubes in boiling water for 2 min or by immersing the tubes in ice water and rapidly adding 0.15 N NaOH to a final concentration of 0.075 N, under which condition the G6PD is inactivated (Table I). The sample was next heated to 60 C for 15 min to destroy the remaining NADP. The induced fluorescence was then determined.

Figure 1 shows that while NADPH is stable at *p*H 7.5 at both 22 and 37 C, it is degraded slightly at 60 and markedly at 80 and 100 C. This effect was observed over an eightfold concentration range at the temperatures tested (Fig. 2).

Table II shows the effect of temperature on NADPH produced in the assay of G6PD. It can be seen that in the range of NADPH generated

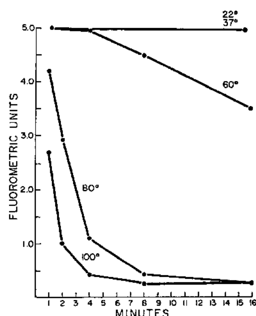

Fig. 1. Effect of temperature on 2×10^{-5} M NADPH in 0.05 M tris–HCl at *p*H 7.5. Aliquots were incubated for 1–16 min at the various temperatures. The fluorescence was determined at room temperature as described in the text.

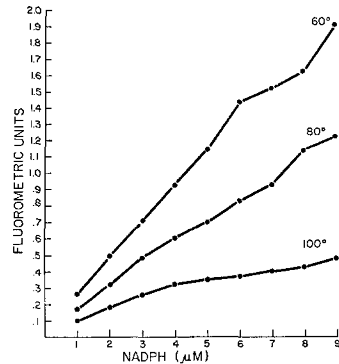

Fig. 2. NADPH fluorescence over an eightfold concentration range in 0.05 M tris–HCl at *p*H 7.5 following 2 min treatment at 60, 80, and 100 C. The fluorescence was determined at room temperature.

Table II. Effect of Various Treatments on NADPH Formed in the Assay of G6PD[a]

	Fluorometric units
Blank	0.30
Ice and dilute NaOH	1.71
60 C for 2 min	1.65
100 C for 2 min	0.80

[a] Yeast G6PD at a concentration of 0.0001 IU/ml of reaction mixture was incubated for 60 min at 37 C, and native fluorescence was read at room temperature following the various treatments. Reaction mixture is the same as described in Table I.

(producing solutions of approximately 3×10^{-6} M) the fluorescence of samples that were boiled was 50% or more lower than that of samples treated with ice and dilute base.

The fluorescence of pyridine nucleotides is being used with increasing frequency for following enzymatic reactions. The highly sensitive procedure of induced fluorescence requires that the reaction first be terminated. In 0.1 N NaOH, NADPH is extremely stable and can withstand boiling for 1 hr (Lowry *et al.*, 1961); however, it is degraded rapidly by high temperature at physiological *p*H, as shown here. An alternate approach for termination of the G6PD reaction would be immersion of the sample in an ice bath, followed by immediate addition of dilute NaOH (Table I).

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