NAD Biosynthesis as an Early Part of Androgen Action

CARL RITTER

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania

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SUMMARY

One 150-μg dose of testosterone stimulates a rise in prostatic NADH of castrate rats within 1 hr. This is followed by a decrease in NADH and an increase in NAD between 1 and 3 hr. Between 1 and 5 hr the sum of NAD + NADH is constant and is more than twice that seen in either the intact control or the castrate control. Between 5 and 10 hr after the injection the NAD + NADH decreases toward the intact control level. The NADPH concentration is about 4 times the castrate or intact control value 2–4 hr after injection. The ATP concentration decreases to a minimum value 1–2 hr after testosterone injection, then increases quickly between 2 and 3 hr, and more slowly between 3 and 9 hr. Neither actinomycin D nor puromycin inhibits the NAD response assayed 8 hr after testosterone injection. The possibility is discussed that androgen activates processes independent of its activation of RNA and protein synthesis, and that part of such activation is a redirection of energy metabolism toward the more efficient production of ATP.

INTRODUCTION

An effect of androgens on sensitive tissues which have been made atrophic by castration is a stimulation of oxidative metabolism (1–5). The enzymic components whose activities are most reduced by castration and restored by androgen injection are those associated with the mitochondrion, for example: malate dehydrogenase and fumarase. The soluble NADP-linked isocitric dehydrogenase is not affected by castration, while both α-glycerophosphate dehydrogenase and lactic dehydrogenase activities are increased as a result of castration (6). The processes of castration and regeneration have been followed, in part, by electron microscopy (7, 8). Castration has been shown to produce a decrease in the number of mitochondria per cytoplasmic area. This effect becomes apparent between 2 and 3 days after castration (8).

Unfortunately, most of the measurements of oxidative capacities of tissues sensitive to androgen have been made days after either castration or the injection of androgen into a castrate animal. In a recent report, however, Wicks and Villee (9) reported an increase in both Q_{O_2} and citric acid secretion in seminal vesicle slices of rats castrated for 4 days and injected with testosterone 18 hr before the seminal vesicles were removed.

A second effect of androgens on sensitive tissues is to stimulate RNA and protein synthesis. In the seminal vesicle of weanling rats (10) and castrate rats (11), the prostate of castrate rats (3, 12) and the kidney of the castrate mouse (13), RNA synthesis proceeds more rapidly after testosterone injection. Such testosterone injection also increases the rate of amino acid incorporation into protein (12–14). Suggestions have been made (15, 16) that the stimulation of RNA synthesis represents an important part of androgen action: that it may involve specific gene unmasking, allowing the synthesis of certain specific messenger RNA molecules. These RNA molecules would presumably be associated

with synthesis of enzymes in which the castrate cell was deficient, and which were required to allow cell restoration to begin. In line with this idea is the finding that one type of RNA made soon after testosterone injection into castrates is nuclear in origin (17, 18).

If the messenger RNA activation hypothesis is true, it is necessary to find enzyme systems which are deficient in androgen-sensitive tissues of the castrate, and which are stimulated by androgen. The enzymes of the mitochondrion seem to represent such a system. It would also seem very logical to have more efficient sources of ATP manufactured as a prime requisite to later recovery processes.

Our approach has been to measure the change in pyridine nucleotide and ATP concentrations during the first hours after injecting testosterone into rats which had been castrated 2 weeks previously. These measurements were made using specific enzymic methods. In addition the *in vivo* change in pyridine nucleotide concentration was measured as a change in the fluorescence emission from the surface of the prostate. Both the change in steady state pyridine nucleotide fluorescence and the increment by which pyridine nucleotide fluorescence was increased after the tissue was made anoxic were measured *in vivo*.

Once the time course of the pyridine nucleotide response was established, experiments were done to determine the sensitivity of the response to an inhibitor of nuclear RNA synthesis, actinomycin D (19), and an inhibitor of cytoplasmic protein synthesis, puromycin (20).

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (180–220 g body wt) were castrated 2 weeks before the intramuscular injection of testosterone (Testryl, Squibb, suspended in water). The dose used was 150 μg per rat (21) in a volume of 0.1 ml actinomycin D (a gift of Merck, Sharpe & Dohme, West Point, Pennsylvania) was suspended in 70% ethanol, and injected intraperitoneally in a 0.1 ml volume just after the testosterone. The concentrations of actinomycin D which were used were 50, 100, 200, 300, and 500 μg/rat (0.25–2.5 μg/g body weight). This dose range was chosen since the response of the more sensitive estrogen-stimulated uterine system had been shown to be fully inhibited by a dose of 500 μg per rat (22, 23). Puromycin dihydrochloride (Nutritional Biochemical Corporation, Cleveland, Ohio) was dissolved in water and injected intraperitoneally just after the testosterone. The doses of puromycin were 10 and 20 mg/rat. These doses 8.5 and 17 μmoles/g were 85–42.5 times greater than the dose used by Liao and Williams-Ashman (12) to inhibit *in vitro* protein synthesis in a system whose ribosomal and soluble components were isolated from the rat prostate. The 20 mg dose is 85 times greater than that which blocked the *in vitro* system (0.2 μmole/ml). Castrates injected with water at the same time as other castrates were injected with testosterone served as castrate controls. Uninjected intact rats served as intact controls. The control rats were sacrificed after the last group of experimental animals, 8–12 hr after the start of an experiment. Testosterone-injected castrates injected with 70% alcohol served as controls for the testosterone-injected castrates injected with actinomycin D. Testosterone-injected castrates given an intraperitoneal injection of distilled water served as controls for the testosterone-injected castrates injected with puromycin.

Analytical and *in vivo* measurement of pyridine nucleotide responses were made at intervals after the testosterone injection. The actinomycin D and puromycin-treated-testosterone injected rats were sacrificed 8 hr after being injected, when the NAD response had become stabilized. Analytical measurements of ATP were made between 15 min and 4 hr after testosterone injection.

Preparation of tissue. Each prostate was exposed and freed of fat and connective tissue by careful dissection while the rat was anesthetized with 50% CO_2, 50% O_2 (24). The prostate was excised, blotted, and then frozen between 2 aluminum blocks precooled with liquid nitrogen. The elapsed

time between tissue excision and freezing was 5 sec or less. Since it required about 5 min to anesthetize each rat and to perform the dissection, and since prostates from 5 rats were used in each determination, each point in the results from such determinations represents the indicated time plus 20–30 min. Analytical results indicating maxima or minima in the concentrations of pyridine nucleotides or ATP were checked in separate experiments 3 or 4 times.

Analytical methods. After the prostates were frozen, a group of 5 for each time interval were pulverized in a Teflon percussion mortar precooled to liquid nitrogen temperature. Half of the pulverized tissue was used for ADP, ATP, and oxidized pyridine nucleotide analyses, and the other half was used for reduced pyridine nucleotide analyses. Pyridine nucleotides, ADP, and ATP were extracted from the frozen powder by the procedures of Williamson,

TABLE 1

Prostrate protein concentrations in castrate and intact controls and in castrates 1–8 hr after the injection of 150 μg of testosterone

Subject	Mg protein/g wet wt
Castrate control	324
2 hr testosterone	268
3 hr testosterone	318
6 hr testosterone	295
7 hr testosterone	279
8 hr testosterone	345
Intact control	277
Mean protein concentration	301 ± 10

and the analyses were done using an Eppendorf fluorometer (25). Recoveries of oxidized pyridine nucleotides, ADP, and ATP were quantitative. Recovery of NADH was 80%, and NADPH, 70%. The results have not been corrected for losses of reduced pyridine nucleotides. The protein content of the prostates was constant in all samples, 30 ± 1% of the wet weight, on the basis of biuret determinations. The constancy of the protein concentration is shown in Table 1.

In vivo determinations. The Ultropak microfluorometer of Chance and Legallais (26) was used as a single beam instrument to measure the intensity of the fluorescence emission stimulated by 366 mμ light from a 1000-watt high pressure mercury lamp (General Electric AH-6). At various times after testosterone injection, each castrate was anesthetized with urethan (0.5 g per rat), a tracheostomy was performed, and the prostate surface was freed of connective tissue. A discrete circular area of the prostate, about 2.0 mm in diameter, was brought into focus, and the rat was immobilized to allow measurements to be made. Total pyridine nucleotide coupled to respiration was determined by allowing the rat to breathe pure nitrogen until respiration stopped, then pure oxygen after respiration resumed, and measuring the change in intensity of pyridine nucleotide fluorescence which resulted. The largest contribution from such a response is thought to come from that pyridine nucleotide which is bound to protein (e.g., that bound to intramitochondrial sites). Such binding results in the intensification of fluorescence from reduced pyridine nucleotide from 2.3 to 5.8 times (27).

The change in pyridine nucleotide redox state *in vivo* was also followed fluorometrically. The castrate rat was prepared as already described, but testosterone was not injected until basal pyridine nucleotide fluorescence was being recorded from the prostate. Then testosterone was injected and the response was followed for several hours.

RESULTS

Figure 1A shows that the prostatic NAD concentration changes in a cyclic manner during the first 12 hr after testosterone is injected into a castrate rat. Maxima which are statistically significant are seen 3–5 and 8 hr after the injection. The NADP concentration is significantly increased only at 8 hr. The 12 hr NAD and NADP concentrations are very close to those found in the prostates of intact controls. Figure 1B shows that the NADH concentration increased about 7 times during the first hour

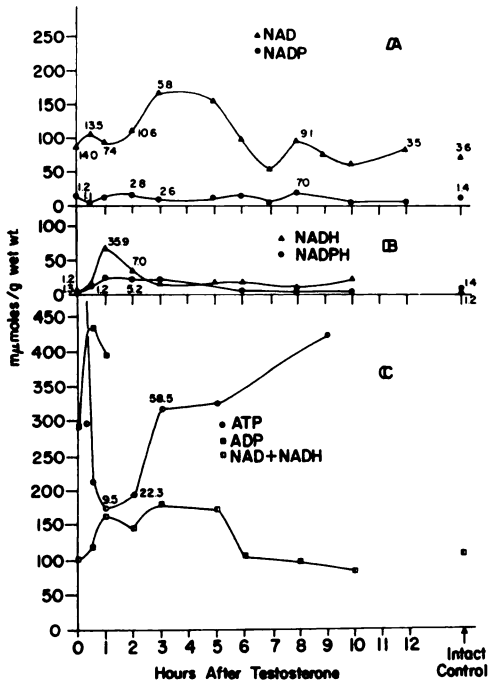

FIG. 1. *Oxidized (▲ NAD, ● NADP, Part A) and reduced (▲ NADH, ● NADPH, Part B) pyridine nucleotide concentrations in prostates of castrate rats (0 hr), castrates given one 150-μg injection of testosterone and sacrificed at the indicated intervals, and intact control rats (points at the right end of the curves)*

Part C of this figure shows oxidized plus reduced NAD ◧, ATP ◐, and ADP ■ concentrations. The intact control value for prostatic ATP (not shown) is 766 mμmoles/g wet weight. The castrate control concentration for ATP (also not shown) is 721 mμmoles/g. The standard error of the mean is indicated beside each point where 3 or more determinations were made. Each determination was done in duplicate and involved prostatic tissue from 5 or 6 rats.

of testosterone treatment. The magnitude of the standard error (written beside the point) indicates the variation in the size of this peak, the means of duplicate analyses in 4 experiments being 175.8, 23.8, 26.4, and 53.6 mμmoles/g. It is possible that this NADH peak results from reduction of NAD formed during the first hour after injection and quickly reduced. After 2 hr the NADH concentration has fallen to a level that is about 4 times that seen in the castrate control. The NADH concentration stays at or below this level for the remainder of the experimental interval. The NADPH concentration rises to about 4 times the castrate control value 1 hr after testosterone injection. It is stable at this level over the 1–3 hr interval, after which it falls to a level about one-half that found in the intact control. Most of the NAD response at 3 hr can be attributed to oxidation of NADH between 1 and 3 hr. About 60 mμmoles/g NADH is oxidized and about 70 mμmoles/g NAD is formed. Some reduction of NAD may occur between 5 and 6 hr, but the ratio of NADH formed to NAD lost is not 1:1. The 8 hr NAD peak could arise partly by NADH oxidation. Between 6 and 8 hr, 25 mμmoles of NADH is oxidized and about 40 mμmoles of NAD is formed. Such oxidation-reduction relationships are not apparent in the case of the NADP–NADPH system, nor do there seem to be oxidation-reduction phase relationships between the NAD–NADH and NADP–NADPH systems. Figure 1C shows that the NAD + NADH peak concentration at 1 hr occurs when the ATP concentration is at a minimum. During the first hour as the ATP concentration decreased about 600 mμmoles/g, the ADP concentration increased about 100 mμmoles/g. Between 1 and 2 hr the cells of the prostate contain maximal reducing power (NADH:: NAD = 0.0). The second NAD + NADH peak, that at 3 hr, occurs when the ATP concentration has attained a plateau. At 3 hr the NADH:NAD ratio is about 0.09, the peak largely due to the contribution made by NAD.

Table 2 shows the effect of various doses of actinomycin D on the concentration of NAD 8 hr after an injection of testosterone. These data indicate that the increased NAD concentration seen at this time in testosterone-injected castrates is not altered by actinomycin. Since the actinomycin did produce such physical signs as an increased volume of peritoneal fluid, bloody exudate in the lumen of the jejunum, and diarrhea with occasional bloody stools, it seems that the population of cells in the mucosa of the small intestine responded to the drug. The rats injected with 70% alco-

TABLE 2

Effect of actinomycin D (0–500 μg/rat) and puromycin (0, 10, and 20 mg/rat) on the concentration of prostatic NAD of castrate rats injected with testosterone and sacrificed 8 hr after injection

Each dose of antibiotic was tested on a group of 5 or 6 rats. The data shown are the means ± the range of duplicate NAD analyses for each group.

Dosage per rat	Prostatic NAD (mμmoles)
Actinomycin D (μg)	
0	81.5 ± 7.0
50	78.4 ± 6.0
100	74.5 ± 0
200	118.0 ± 3
300	92.8 ± 0
400	82.0 ± 0
500	86.6 ± 7
Puromycin (mg)	
0	95.0 ± 9.0
10	93.0 ± 20.0
20	99.9 ± 9.0

hol (actinomycin D controls) did not have these symptoms. These findings seem to indicate that the actinomycin was effective. If this is so, and if the cells of the prostate were affected as were those of the small intestine, then perhaps *de novo* enzyme synthesis in response to testosterone was not necessary for the pyridine nucleotide and ATP responses which occurred after testosterone injection.

Table 2 also shows that massive amounts of puromycin injected immediately after testosterone had no effect on the NAD response 8 hr after injection. Thus the response seems to be independent of protein synthesis originating from messenger RNA manufactured during *post-castration atrophy*.

Figure 2 shows that the increased pyridine nucleotide reduction can be recorded *in vivo* with the Ultropak microfluorometer. Parts A and B of this figure show the response from ventral prostates of two

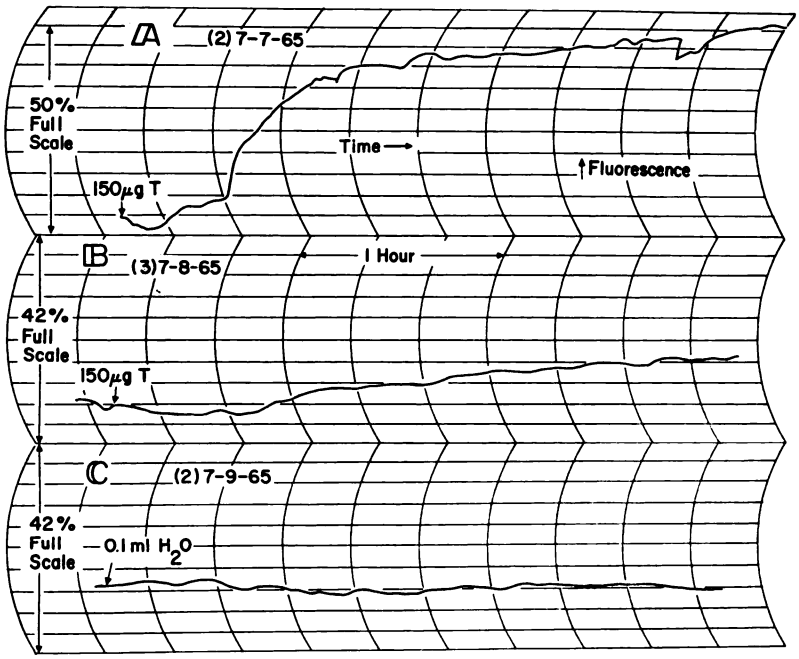

FIG. 2. *Change in pyridine nucleotide fluorescence in the ventral prostate of the castrate rat in response to testosterone*

Parts A and B show the effect of testosterone (T) injection on 2 different rats. Part C shows a control fluorescence tracing from the prostate of a rat injected with water (the vehicle in which the testosterone was suspended). The full width of each record is shown, as is the relative sensitivity used for each experiment.

castrate rats given testosterone (abbreviated *T* on the figure). Part C shows that the increased fluorescence is not seen in a castrate injected with water (the vehicle in which the testosterone was suspended).

In part A of Fig. 2 pyridine nucleotide fluorescence begins to increase 30 min after testosterone injection, and the rise in fluorescence to a maximum level is essentially complete within 50 min. Part B of Fig. 2 shows that the process of increased pyridine nucleotide reduction did not begin until 40 min after testosterone injection and did not go to a fairly stable level, but kept increasing. These curves of changing pyridine nucleotide fluorescence emphasize the quantitative differences between animals as regards both the intensity of fluorescence (amount of pyridine nucleotide reduced) and the time when the increase in fluorescence began. These curves confirm both the increase in reduction of pyridine nucleotide about 1 hr after testosterone injection and that the amount of reduction is variable from animal to animal. However, the analytical results indicate that the peak pyridine nucleotide reduction gives way to a highly oxidized state in the prostate. Evidence for such reoxidation was not seen in this series of *in vivo* experiments involving a total of 8 castrate rats injected with testosterone.

Figure 3 is a copy of an experimental record showing a cycle of pyridine nucleotide fluorescence from the surface of the ventral prostate of a castrate rat 5 hours after the injection of 150 μg of testosterone in response to a nitrogen oxygen cycle. The apnea and resulting tissue anoxia produced by the inspiration of nitrogen caused an increase in the intensity of fluorescence emission above that seen when the rat was breathing either air or oxygen. No change in fluorescence intensity occurred until the animal stopped breathing. Then there was a cycle of increased fluorescence and a return of the fluorescence to that seen prior to the apnea. Then the animal started breathing, with little further change in fluorescence intensity. This type of cycle was reproducible from animal to animal, while the usual type of N_2–O_2 cycle (28), used with kidney, liver, and brain, where the animal breathes N_2 and pyridine nucleotides become reduced, then the animal breathes O_2 and pyridine nucleotide oxidation occurs, was not useful in the case of the prostate. The animals stopped breath-

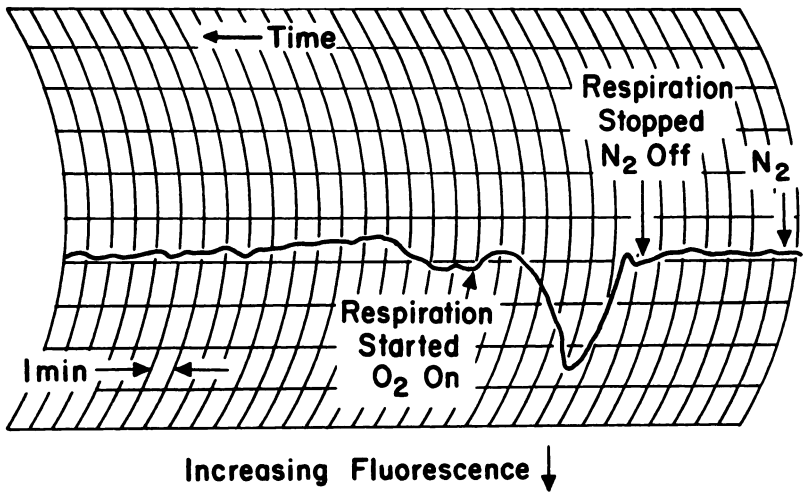

FIG. 3. *Changes in pyridine nucleotide fluorescence recorded from the surface of the prostate in a castrate rat which had been injected 5 hr previously with 150 μg of testosterone*

Fluorescence increased sharply about 0.5 min after apnea, then decreased slowly to the point where respiration resumed. The resting fluorescence level was reached 10 min after breathing resumed.

ing before nitrogen inspiration produced any significant effect in the redox state of the prostatic pyridine nucleotide. It may be that the cycle shown in Fig. 3 is largely a result of homeostatic vasomotor changes in response to the inspired nitrogen. Thus the prostatic anoxia leading to increased fluorescence intensity may be due to vasoconstriction, and subsequent vasodilation may result in the oxidation of that pyridine nucleotide which had been reduced. The parameter which was taken as an index of total pyridine nucleotide linked to respiration was the largest increase in fluorescence recorded during the first N_2–O_2 cycle from each animal.

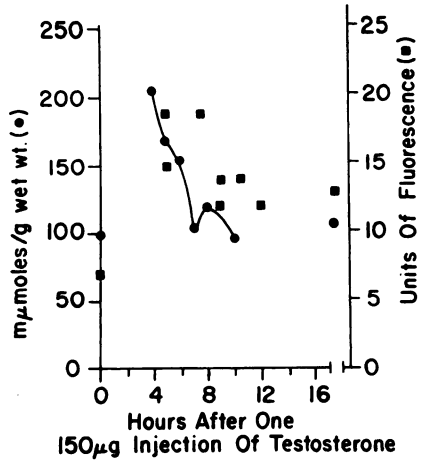

FIG. 4. *Correlation of increment of the increased fluorescence intensity obtained during the first N_2—O_2 cycle from the prostates of testosterone-injected castrates* (■) *with the total pyridine nucleotide present, determined analytically* (●)

The analytical data are taken from Fig. 1. Castrate control values are shown as points close to the right ordinate.

Figure 4 shows a comparison of the *in vivo* fluorescence of total pyridine nucleotide linked to respiration with the total pyridine nucleotide determined analytically. Each square in this figure represents the change in fluorescence during the first nitrogen-oxygen cycle from one animal. The point on the left ordinate is from the prostate of a castrate control rat, while that at the end of the curve is from an intact control. The analytical pyridine nucleotide data are from Fig. 1. Despite the scatter of the points from the *in vivo* experiments the substances being measured by both techniques are decreasing in concentration similarly over the 4–10 hr period after testosterone injection. This comparison shows that the changes in prostatic pyridine nucleotide concentrations in response to testosterone are likely to be real. It is also likely that the largest contributor to the response, especially during the last 6 hr, was NAD, and that, therefore, the NAD measured analytically was not formed from NADH during tissue preparation and extraction.

DISCUSSION

The earliest response of the prostatic pyridine nucleotide concentration was a small increase in NAD during the first half hour. Between ½ and 1 hr NADH increased about 60 mμmoles/g while NAD decreased only about 20 mμmoles/g. During the first half hour the main testosterone-stimulated process seems to be NAD biosynthesis. During the second half hour both NAD reduction and biosynthesis are probably occurring. After this first hour about 170 mμmoles/g of NAD + NADH is present, whereas in the castrate control about 70 mμmoles/g was present. This level of about 170 mμmoles of NAD + NADH is maintained from 1 to 5 hr after injection. After 5 hr this sum begins to decrease, and the fall is not associated with stoichiometric increases in NADP or NADPH. This would seem to rule out any large-scale transhydrogenase activation by androgen as being important during the first 10 hr of the testosterone-stimulated response. Such a conclusion has also been reached by Wicks and Villee for longer times after androgen injection (9). Since the protein concentrations of the prostates were constant during the experimental interval, the decrease in NAD + NADH cannot be explained by increased prostatic water content. It seems more likely that the rise in NAD + NADH between 0 and 1 hr after testosterone injection and its maintenance for the first 5 hr after such injection is

related to alteration in the scheme of energy metabolism from that found in the castrate prostate to that found in the intact control prostate. The concentrations of pyridine nucleotides are above castrate and intact control levels only during the period from ½ hr to 6 hr after testosterone injection.

While the concentrations of pyridine nucleotide in the castrate and intact control prostates are very similar, there is considerable evidence that the energy metabolism in prostates from the two types of control rats is very different. The energy metabolism of the prostate in the castrate control is probably largely based on aerobic glycolysis. This supposition is based on work done by Levey and Szego (4, 5), Rudolph and co-workers (1, 2), and by Williams-Ashman (6). It is supported by the occurrence of maximum NADH concentration and minimum ATP concentration in our results, 1 hr after testosterone injection. This is the sort of ATP::NADH phase relation which is seen in an oscillating system of aerobic yeast cells, whose main mechanism of ATP production is via aerobic glycolysis (29). It is also likely that some ATP is used during the first hour for NAD synthesis (30, 31).

During the second and third hour after testosterone the prostate becomes highly aerobic. This is indicated by the low ratio of NADH:NAD. The ATP level increases very rapidly during the 2–3 hr interval. Since most of the synthesis of NAD is complete, it is likely that this ATP will be or is being used for RNA synthesis, if there is a close correlation between ATP synthesis and RNA synthesis such as that found by Feigelson and Feigelson (32) in livers of cortisone-treated rats. This rapid rise in ATP concentration seems to indicate that either the rate of ATP production via glycolysis is proceeding very much faster, or that a new source of ATP has come into play. Electron microscopic data lead us to favor the later possibility (7, 8). This is supported by our finding of increased pyridine nucleotide linked to respiration as soon as 4 hr after testosterone injection, a time at which the low NADH:NAD ratio, seen first at 3 hr, is maintained. It is also supported by the finding that the intact control prostate, containing very nearly the same concentrations of all 4 pyridine nucleotides as does the prostate 6–10 hr after testosterone, has a much more active oxidative metabolism than does the castrate control prostate (1, 2, 6). The activation of oxidative metabolism has been known to occur within 18 hr after an injection of testosterone (1, 2, 9). The data presented in this paper indicate that this activation occurs during the first hour after testosterone injection.

The biosynthesis of NAD seems to be independent of nuclear RNA synthesis or nucleocytoplasmic RNA efflux (19). However, further study will be necessary to prove that the concentrations of actinomycin D which we used were effective in inhibiting RNA synthesis. It is of interest that Hamilton (22) was able to block estrogen-stimulated RNA synthesis in the uteri of castrated female rats with a dose of 500 μg actinomycin D per rat and that Chambers *et al.* (33) were able to block the induction of rat liver tryptophan pyrrolase activity with about 250 μg per rat. In our experiments the actinomycin may actually have stopped RNA synthesis so well that significant ATP was rechanneled from RNA synthesis into NAD synthesis. We have shown that concentrations of actinomycin between 100 and 400 μg/rat produced concentrations of NAD 8 hours after testosterone injection that were from 10 to 25 mμmoles/g more than were expected. The actinomycin and the puromycin experiments seem to show that the NAD response can proceed in a system where protein synthesis is at least partly inhibited. This type of response would have been expected if testosterone had stimulated several metabolic pathways, rather than just stimulating RNA synthesis (15, 16).

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