within 72 hours after exposure to cold. The plasma PBI in Group II was lower than in Group I and higher than in Group III. This suggests that the hamster likely has ectopic thyroid tissue which enhances survival of thyroidectomized animals. A comparison of control and surgically-ThX animals suggest that the known increases in TSR at cold temperatures are not significant in survival to extreme cold.

Further investigations should test the value of thyroxine replacement of survival of the radio-thyroidectomized animals and a comparison on propyl thiouracil and thyroxine-supplemented intact animals when exposed to extreme cold.

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Studies on Phosphorylase Activity in the Rat Ductus Deferens* (32815)

SAMUEL L. LEONARD
Division of Biological Sciences, Cornell University, Ithaca, New York 14850

In skeletal muscle, low levels of phosphorylase *a* activity are obtained when manipulative stimulation of the tissue is kept minimal prior to assay and when ethylenediaminetetraacetic acid (EDTA) is employed in the homogenizing medium which prevents the *in vitro* conversion of phosphorylase *b* → *a* by inhibiting phosphorylase *b* kinase (1). The extent to which these factors affect phosphorylase *a* activity in the smooth muscle of the rat ductus deferens has not been clearly established. Phosphorylase *a* activity determined shortly after removal of the ducts from rats was eight times greater than that found in ducts placed in a warm, aerated, saline bath for 30 min prior to assay (2). Treatment with EDTA had no effect on levels of phosphorylase *a* activity determined in these ducts (3). The results of further studies on the effects of manipulation and EDTA on phosphorylase *a* activity in the rat d. deferens will be presented.

Methods. Large adult intact rats and some castrated for 6 days were used. The procedure for removal of the ducts and trimming before freezing with solid CO_2 and the method of suspending them in Krebs–Ringer–bicarbonate–glucose medium, gassed with 95% O_2, 5% CO_2 were described previously (2). To remove, trim, and express the sperm from each duct requires 35–40 sec and these ducts are designated as "manipulated." Ducts removed and frozen with solid CO_2 immediately (within 3–5 sec) then trimmed in the frozen state before assay, are designated "quick-frozen." When ducts were to be stimulated electrically, they were suspended on hooks (2), the electrodes placed at the ends of the ducts and shocks given for 10 sec (30 V/

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20 millisec duration/20 per sec). They were frozen immediately thereafter. A single dose of CaEDTA (0.6 mmole/kg, pH 7.3) was injected intraperitoneally 1 hour before autopsy in some experiments.

The assay procedure for phosphorylase determinations used in most experiments has been described (4) as modified for the d. deferens (2). In some experiments, one duct was homogenized in a medium containing 0.1 *M* NaF plus 1.2 $\times$ 10^{-3} *M* NaEDTA, pH 6.1, and the other in 0.1 *M* NaF alone (control). The results are given in μg of P formed by 100 mg of tissue in 10 min at 37°C with standard errors of the means.

A different assay procedure was also introduced, based on one described for studies on uterine phosphorylase (5). One frozen duct was homogenized in an ice-cold medium containing the following solutions in proportions of 1:1:2; 2% glycogen, 0.058 *M* glucose-1-PO_4, pH6.1, and 0.1 *M NaF*. The other was homogenized in a similar medium except 1.2 $\times$ 10^{-3} *M* NaEDTA, pH6.1, was combined with the NaF. Approximately 50 mg of tissue was used and the homogenate adjusted so that 1 ml contained 7 mg of tissue. A schedule was rigidly followed: 4 min for grinding, diluting, and placing in a 37° bath; and after 2 min of warming, a 0.5-ml sample was removed and added to a tube containing 0.5 ml of 10% cold trichloroacetic acid. This was diluted with water immediately and the tubes kept in an ice bath. Additional samples were taken at intervals and the P liberated determined in all the samples as described in the first assay method (2,4). The value for the first sample was assumed to be zero time reaction blank and was subtracted from all other values. The cumulative P liberated at each interval, representing phosphorylase *a* activity, was calculated per 100 mg of tissue plotted against time. Omission of glucose-1-PO_4 or glycogen from the reaction resulted in no appreciable gain in P.

Results. In order to determine if "manipulation" of the ducts was inducing maximum phosphorylase *a* activity, one duct from each intact rat was electrically stimulated immediately after trimming and the contralateral one was not. Apparently the manipulation of the ducts in preparation for freezing induced a maximum increase in phosphorylase *a* activity which was not further enhanced by shocking (Table I). When both "manipulated" ducts were placed *in vitro* for 30 min, the one which received the electric shock showed a marked increase in phosphorylase *a* activity above the control level (Table I). Total phosphorylase *t* (assayed in the presence of adenylic acid) and *a* activities were lower after incubation than they were initially. The changes in the activity ratios *a/t* reflect the marked changes in phosphorylase *a*.

TABLE I. Phosphorylase Activity in the D. Deferens: Effects of Electrical Stimulation and Castration.

Stimulation[a] (+ or 0)	Phosphorylase (μg P/100 mg of tissue $\pm$ SE)		
	a	*t*	*a/t* $\times$ 100
Intact rats			
Immediate, +	520 $\pm$ 28	831 $\pm$ 45	62.6 $\pm$ 1.5
0	510 $\pm$ 22	821 $\pm$ 12	62.2 $\pm$ 0.8
Delayed, +	109 $\pm$ 8[b]	618 $\pm$ 48	18.0 $\pm$ 1.7[b]
0	59 $\pm$ 7	667 $\pm$ 13	8.8 $\pm$ 1.0
Castrates			
Immediate, +	296 $\pm$ 12	526 $\pm$ 19	53.6 $\pm$ 0.9
0	263 $\pm$ 20	515 $\pm$ 25	50.8 $\pm$ 2.0
Delayed, +	76 $\pm$ 4[b]	485 $\pm$ 17	15.7 $\pm$ 0.9[b]
0	38 $\pm$ 3	461 $\pm$ 26	8.3 $\pm$ 0.5

[a] Immediate, stimulated after dissection; delayed, stimulated after 30 min *in vitro*.

[b] $p < .01$, others not significantly different at this level; at least 4 rats per experiment.

The experiment was repeated employing ducts from castrated rats, and the results obtained were similar to the previous ones. The values for phosphorylase activity in ducts from the castrates were less than those from intact rats, confirming previous observations (2).

To circumvent the *in vitro* treatment of the ducts in order to obtain low levels of phosphorylase *a* activity, a "quick-freeze" technique was used. One duct from each of 4 intact and 4 castrated rats was "quick-frozen" and the other "manipulated" before freezing. In the intact rats, phosphorylase *a* activity from quick frozen and the manipulated ducts was 119 $\pm$ 18 and 391 $\pm$ 27 μg of P,

TABLE II. Effect of EDTA on Phosphorylase Activity in Quick Frozen D. Deferens from Intact and Castrated Rats.

Experiment EDTA (+ or 0)	Phosphorylase (μg P/100 mg of tissue $\pm$ SE)		
	a	*t*	$a/t \times 100$
EDTA in assay			
Intact rats, +	334 $\pm$ 18	620 $\pm$ 21[a]	53.8 $\pm$ 1.8
0	129 $\pm$ 12	604 $\pm$ 30	21.3 $\pm$ 1.5
Castrates, +	155 $\pm$ 10	384 $\pm$ 14[a]	40.3 $\pm$ 1.1
0	65 $\pm$ 7	382 $\pm$ 12	16.8 $\pm$ 1.3
EDTA injected			
Intact rats, +	249 $\pm$ 6	596 $\pm$ 19[a]	42.1 $\pm$ 2.0
0	152 $\pm$ 11	603 $\pm$ 13	25.2 $\pm$ 1.6
Castrates, +	164 $\pm$ 8	426 $\pm$ 29[a]	38.9 $\pm$ 2.2
0	92 $\pm$ 12	447 $\pm$ 12	20.6 $\pm$ 2.9

[a] Differences not significant, all others $p < .01$; at least 4 rats per experiment.

respectively. In castrated rats, the enzyme activity from quick-frozen and the manipulated ducts was 50 $\pm$ 5 and 119 $\pm$ 15 μg of P, respectively. The total phosphorylase *t* activities were not influenced by the method of handling the ducts from either intact or castrate rats although lower values for phosphorylase *t* in the ducts from castrates compared with those from intact rats were observed. Data on phosphorylase *t* are not presented in these preliminary observations.

If manipulation of the ducts before freezing produced what appeared to be maximum activation of phosphorylase, then manipulation could possibly mask any increases in enzyme activity produced by other means. Since EDTA treatment failed to produce a significant effect on phosphorylase *a* activity in ducts prepared by first "manipulating" then freezing (3), experiments with EDTA were made using the "quick-freezing" technique. Ducts from intact and castrated rats were employed and the effects of EDTA were observed either by adding the reagent to the homogenizing medium or by injecting it in the form of CaEDTA. The EDTA was not added to the homogenizing medium when ducts from injected rats were employed. The results in Table II show that when EDTA was present in the homogenizing medium, or when the ducts were from rats injected with CaEDTA, phosphorylase *a* activity was always greater than in the controls. Phosphorylase *t* activity was not affected by EDTA.

A different assay procedure for phosphorylase *a* activity was introduced in which the accumulated P produced was determined at timed intervals in ducts, manipulated, and quick frozen, and the assays made with and without EDTA. Ducts from 4 intact rats were manipulated and those from 4 other rats were quick-frozen. One duct from each rat was homogenized with EDTA and one without EDTA. The results within each group of rats were similar and the data are pooled in Fig. 1.

When EDTA was present, phosphorylase *a* activity was essentially linear throughout the period of the assay. In the absence of EDTA, the enzyme activity soon began to decrease so that less inorganic P accumulated with time as indicated by the curved lines in Fig. 1.

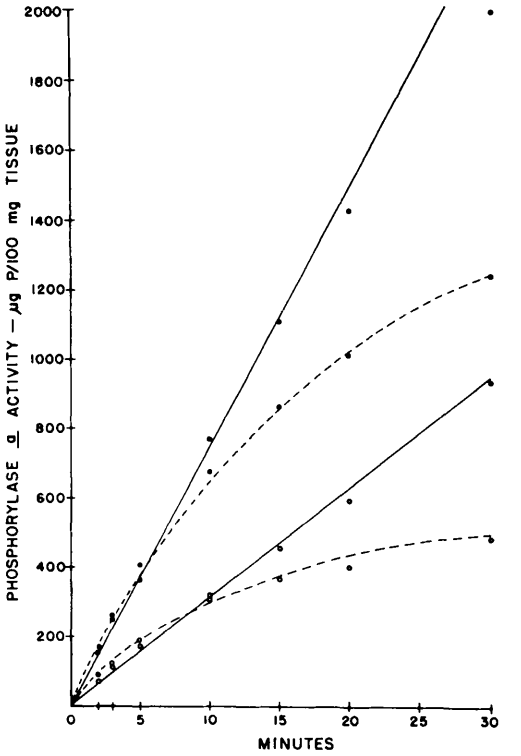

FIG. 1. Phosphorylase a activity in d. deferens shown by the amount of P accumulated at intervals during incubation. Assay with EDTA (———), without EDTA (- - - -); ●, manipulated ducts, ○, quick frozen ducts.

Evidence for greater enzyme activity in the manipulated ducts vs those quick frozen is also noted.

Discussion. The experiments show that trimming the d. deferens and expressing the residual sperm comprise sufficient stimulation to increase phosphorylase *a* activity maximally. Electric shocks to the ducts after manipulation did not increase the enzyme activity further although this stimulation was sufficient to increase phosphorylase *a* activity above an initially lower base level obtained in the ducts *in vitro*. An extremely high level of phosphorylase *t* activity was observed in manipulated ducts of intact rats (Table I). If it is assumed that the rapid increase in phosphorylase *a* activity was the result of a conversion from inactive phosphorylase *b*, the total activity should not change. This high level of total activity is not explained.

There was no marked difference in the type of response in the ducts from intact and castrated rats but the effect of castration in lowering phosphorylase activity confirmed previous observations (2).

The advantage of "quick-freezing" the ducts before assaying is to obtain a lower and presumably more nearly normal level of phosphorylase *a* activity which might be expected of tissue at rest *in situ*. The lowest levels of phosphorylase *a* activity were observed when the ducts were resting *in vitro* and frozen immediately upon removal from the bath. In skeletal muscle, the less the disturbance and quicker the freezing before assay, the lower is the level of phosphorylase *a* activity (1).

When EDTA was either injected into the rats or added to the homogenizing medium and the ducts assayed by the original method, phosphorylase *a* activity levels were always higher than in the controls. The original report of the effects of EDTA in increasing this enzyme activity were made in rat uterine smooth muscle (3). In contrast, the effect of EDTA in the determination of phosphorylase *a* activity in skeletal muscle has been to give lower values, whether it was injected (3) or present during homogenization (6). The EDTA acts by binding divalent cations necessary for phosphorylase *b* kinase to convert phosphorylase $b \rightarrow a$ (6).

By using a different assay procedure, the effects of EDTA on the enzyme activity in relation to handling of the ducts can be explained. The essential feature of this method is measuring the inorganic P accumulated at intervals during the reaction. When EDTA was present, phosphorylase *a* activity was linear and the activity did not diminish with time. In the absence of EDTA phosphorylase *a* activity decreased with time and this is represented by the curved lines in Fig. 1. The EDTA in some way prevented the loss of phosphorylase *a* activity from high initial levels as a result of manipulation of the ducts before assay or from low initial levels in the quick-frozen ducts. If one observes the amount of P accumulated at 20 min incubation (Fig. 1), it would be concluded that EDTA increases phosphorylase *a* activity but at 10 min of incubation this conclusion would not be so readily discernible. This explanation for the effects of EDTA in prevention of the loss of phosphorylase *a* activity was first made in studies with rat uterine smooth muscle (5), and it can now be applied to the d. deferens.

Summary. Maximum phosphorylase *a* activity is induced in the d. deferens of intact and castrated rats during trimming and handling prior to assay and low levels of activity obtain following quick freezing after removing the ducts or after incubating them *in vitro* before freezing. The EDTA, either injected or added to the homogenates *appears* to increase phosphorylase *a* activity. The EDTA prevents a loss of phosphorylase *a* activity in the homogenate which normally occurs when it is not present. This can explain the apparent activating effect of EDTA on phosphorylase *a* in this tissue.

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