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TESTOSTERONE AND SUGAR TRANSPORT IN LEVATOR ANI MUSCLE OF RAT

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SUMMARY

1. The effect of testosterone on xylose transport kinetics in levator ani muscle of sexually immature rat has been studied and compared with those effects of other experimental conditions responsible for changes in penetration of sugar into striated muscle fibers.

2. Testosterone increases the penetration rate of xylose by stimulation of transport mechanisms. This is inhibited by phlorizin. No changes in the penetration of L-glucose take place.

3. Kinetic analysis shows that increased penetration rates are attributable to a considerable increase in v_{max}; K_m values remain substantially unchanged.

4. Such increased penetration rates are not additive to those of insulin, anaerobiosis, direct stimulation and cyanide.

5. The effect of testosterone on penetration of sugar is not dependent on pancreatic, adrenal or hypophyseal secretions. However, acute denervation of the muscle completely blocks action of testosterone.

INTRODUCTION

Sugar transport in muscle takes place through stereospecific mechanisms[1,2] and is under hormonal[3] and functional regulation[4]. Some experimental conditions influencing energy metabolism[3] can also increase the transport rate.

Uptake is the first and, in most cases, the limiting step of sugar utilization by the muscle cell[3], and therefore the study of the influence of hormones on penetration rate of sugar may be of interest in explaining hormonal action on glucose metabolism. It has been recently shown that treatment with testosterone increases both the penetration rate of sugar[5–8] and glycogen synthesis in rat levator ani muscle[7], but only the effect on muscle glycogen content has been found to be additive with that of insulin[8].

Therefore, it was thought interesting to study further the effect of testosterone on uptake of xylose and to compare its action with that of other experimental conditions which can enhance penetration rate of sugar *in vitro*. In order to test whether the effect of testosterone is direct or mediated by other mechanisms, experiments on diabetic, hypophysectomized, adrenalectomized or denervated animals were also performed.

MATERIALS AND METHODS

Animals

Random groups of immature male rats, Wistar–Glaxo strain (50 g body wt.), received maximal doses (5 mg, *cf.* ref. 6) of testosterone propionate 24 h before the experiment.

Fasting was begun at the 12th h after injection.

Diabetes was produced by means of a single intravenous injection of alloxane (60 mg/kg)[9] 3 days before sacrifice. Only highly glycosuric rats (glycosuria was checked by means of TES–TAPE Lilly) were used.

Adrenalectomy by the dorsal approach was performed 2 days before killing the animals. Rats had free access to physiological saline.

Experiments on hypophysectomized rats were carried out on Sprague–Dawley male rats of the same weight (generously donated by Ormonoterapia Richter, Milano); unoperated rats of the same strain were used as controls.

Muscle denervation was obtained by destroying lumbosacral spinal cord 2 h before the intramuscular administration of testosterone propionate. Preliminary experiments showed that this procedure was able to cause a rapid and marked atrophy in the levator ani muscle.

Levator ani muscle was removed under Nembutal anaesthesia (5 mg/100 g body wt.). The intact preparation method was employed (*cf.* ref. 10).

Incubation in vitro

Incubations were carried out at 37° in 2 ml of Krebs–Ringer bicarbonate medium (pH 7.4) gas O_2–CO_2 (95:5, v/v) either in Warburg flasks or in a Dubnoff apparatus with agitation. Incubation time varied in accordance with the type of the experiment being performed.

The incubation medium contained pyruvate (10 mM), inulin (7.5 mg/ml) and xylose (usually 15 mM); 0.25 μC/ml uniformly ^{14}C-labeled xylose was included. Bovine insulin (0.1 unit/ml) or NaCN (1 mM) was added to some incubations.

The apparatus and method of ARVILL[11] were used for direct stimulation *in vitro*. Warburg flasks with white phosphorus in the central well were used to obtain anaerobiosis under a 95:5 (*v/v*) gas mixture of N_2 and CO_2.

Determination of the penetration of sugar

After incubation the muscle was quickly rinsed in a small quantity of inulin and xylose-free Ringer solution, was gently blotted on filter paper, was separated from the bulbocavernosus and was weighed.

Sugars were then extracted in boiling water according to the method of EICHORN AND HECHTER[12]; this method was found as effective as homogenization in trichloroacetic or $HClO_4$ (ref. 8).

Aliquots of the extract were used for the determination of inulin[13]; a further aliquot was spread over No. 3 Whatman paper disks, dried and counted with a Model 725 Nuclear scintillation counter (to less than 1% error). The results are corrected for counting efficiency.

The data are usually expressed as radioactivity distribution ratios between intracellular water and incubation medium, as follows: Distribution ratio = [(total

muscle counts/min) — (incubation medium counts/min per μl) × (extracellular space μl)]/[(μl intracellular space) × (counts/min per μl incubation medium)].

The extracellular space was taken as the inulin distribution space, *i.e.*, total muscle inulin content (corrected for muscle fructose content) divided by the inulin concentration of the incubation medium.

On other occasions, the results are expressed as μmoles sugar transported into the intracellular water of 100 mg of wet muscle. Intracellular water was determined by subtracting extracellular water values from the total muscle water content; the latter was found to be 80 % of the wet weight, as reported by ARVILL AND AHRÉN[10].

Materials

Testosterone propionate (pure or in an oil solution) was kindly donated by the Vister Co. (Casatenovo Brianza) and bovine crystalline insulin having a low glucagon content by the Lilly Co. (Florence).

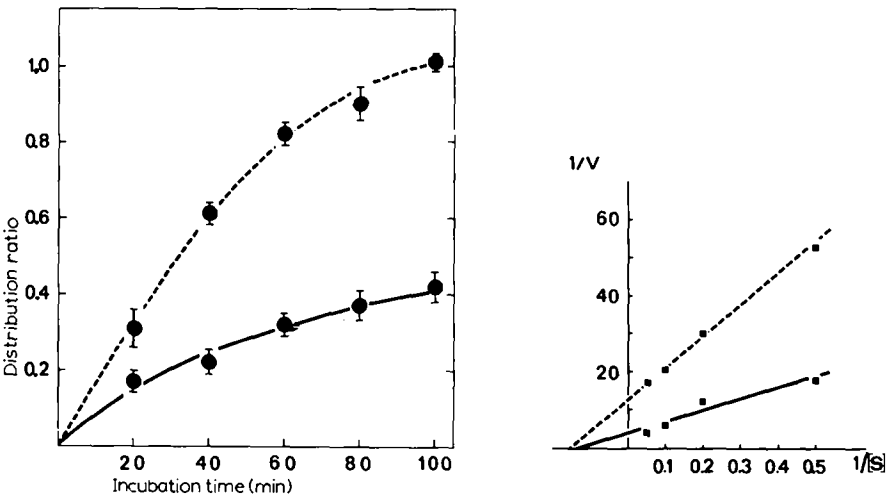


Fig. 1. [$^{14}C_6$]Xylose uptake *in vitro* in levator ani muscle of rats treated (●---●) or untreated (●—●) with testosterone propionate as a function of incubation time. Experiments were performed in Krebs–Ringer bicarbonate medium containing xylose (15 mM), pyruvate (10 mM) and inulin (7.5 mg/ml), under aerobiosis at 37°. Each point represents the mean of five cases. Vertical bars represent 2 × S.E. Incubation time was corrected for the time required to extracellular space equilibration. This was found identical in muscle of rats treated and untreated with testosterone (8 min). Statistical analysis, performed by Prof. G. Segre, by fitting to the data the equation $y = a\ (1 - e^{-kt})$ (sec ref. 33) showed that the best fitting curves are $y = 0.465\ (1 - e^{-0.016t})$* for controls and $y = 1.00\ (1 - e^{-0.014t})$** for testosterone-treated animals.

* $a = 0.465 \pm 0.047$; $k = 0.016 \pm 0.0019$.

** a value was limited in order to exclude sugar concentration in intracellular water; $k = 0.014 \pm 0.0018$. Whereas asymptotic values (a) are significantly different, k values are not.

Fig. 2. Xylose uptake in levator ani muscle of rats treated (■—■) or untreated (■---■) with testosterone propionate as a function of sugar extracellular concentration. Incubation in Krebs–Ringer bicarbonate medium containing pyruvate (10 mM) under aerobiosis for 30 min at 37° in the presence of [$^{14}C_6$]xylose (0.08 mM) and of different amounts of unlabeled D-xylose. The reciprocals of the external concentrations are plotted against the reciprocals of the amount of the sugar transported by 100 mg wet muscle per min, according to Lineweaver and Burk by the least square method. The incubation time was corrected by subtracting the time required for the equilibration of extracellular space (see Fig. 1). Each point is the mean value of 4 cases. [S], mM; v, mmoles × 10^{-1}/min per 100 mg wet wt.

[$^{14}C_6$]Xylose (specific activity, 3 mC/mmole) was supplied by the Radiochemical Centre (Amersham).

All the products used were of analytical grade.

RESULTS

Testosterone treatment increases the penetration rate of xylose and distribution space (Fig. 1). The penetration curve as a function of time shows that the asymptotic value approached by the sugar distribution is almost doubled by the hormone.

Since sugar transport is the result of stereospecific mechanisms, without concentration in the intracellular water[1,2], the question whether the effect of testosterone was due to increased active transport or to nonspecific increases in sarcolemma permeability was investigated. Table I shows that the distribution of L-glucose, a non-actively transported sugar, is unaffected by the hormone. It has also been found that the presence of glucose inhibits xylose transport in both testosterone-treated and control muscles (Table II); phlorizin completely blocks extra accumulation of xylose in the treated muscle (Table II).

Subject to the limits imposed by the experimental conditions (*cf.* ref. 14), study

TABLE I

L-GLUCOSE UPTAKE IN LEVATOR ANI MUSCLE OF RATS TREATED OR UNTREATED WITH TESTOSTERONE PROPIONATE

Incubation in Krebs–Ringer bicarbonate medium containing pyruvate (10 mM) under aerobiosis for 60 min at 37° in the presence of L-[$^{14}C_1$]glucose (0.25 μC/ml). Means of 7 cases ± S.E. are given.

Treatment	L-*Glucose distribution ratio*
Controls	0.13 ± 0.016
Testosterone	0.11 ± 0.018

TABLE II

GLUCOSE AND PHLORIZIN EFFECT ON XYLOSE UPTAKE *in vitro* IN LEVATOR ANI MUSCLE OF RATS TREATED OR UNTREATED WITH TESTOSTERONE PROPIONATE

Incubation medium: see Fig. 1. Experiments were carried out 40 min at 37°. Means ± S.E. are given.

Treatment with testosterone in vivo (mg)	*Treatment in vitro*	*Number of cases*	*Xylose distribution ratio*
0	—	8	0.17 ± 0.020
0	Phlorizin (3 mM)	8	0.19 ± 0.047
5	—	8	0.44 ± 0.041
5	Phlorizin (3 mM)	8	0.23 ± 0.032
0	—	4	0.18 ± 0.031
0	Glucose (11 mM)	4	0.13 ± 0.021
5	—	4	0.45 ± 0.049
5	Glucose (11 mM)	4	0.35 ± 0.050

TABLE III

EFFECT OF INSULIN, ANAEROBIOSIS, DIRECT STIMULATION AND CYANIDE ON XYLOSE UPTAKE *in vitro* IN LEVATOR ANI MUSCLE OF RATS TREATED OR UNTREATED WITH TESTOSTERONE PROPIONATE

Experiments were performed as described in Fig. 3, but preincubation was omitted and incubation was carried out at 37° for 40 min. Means ± S.E. are given. All treatments significantly increased xylose uptake ($P<0.01$). Interactions between testosterone effect and treatments *in vitro* were always significant. ($P<0.01$; for cyanide only $P<0.05$.)

Treatment with testosterone in vivo (mg)	*Conditions in vitro*	*Number of cases*	*Xylose distribution ratio*
0	Insulin (0)	4	0.41 ± 0.044
0	Insulin (0.1 unit/ml)	4	0.76 ± 0.070
5	Insulin (0)	4	0.53 ± 0.023
5	Insulin (0.1 unit/ml)	4	0.70 ± 0.014
0	Anaerobiosis (−)	5	0.38 ± 0.013
0	Anaerobiosis (+)	5	0.59 ± 0.060
5	Anaerobiosis (−)	5	0.73 ± 0.027
5	Anaerobiosis (+)	5	0.77 ± 0.046
0	Stimulation (−)	4	0.22 ± 0.024
0	Stimulation (+)	4	0.59 ± 0.060
5	Stimulation (−)	4	0.71 ± 0.085
5	Stimulation (+)	4	0.79 ± 0.036
0*	Cyanide (0)	4	0.18 ± 0.025
0*	Cyanide (1 mM)	4	0.34 ± 0.040
5*	Cyanide (0)	4	0.45 ± 0.057
5*	Cyanide (1 mM)	4	0.56 ± 0.066

* Incubation time 30 min.

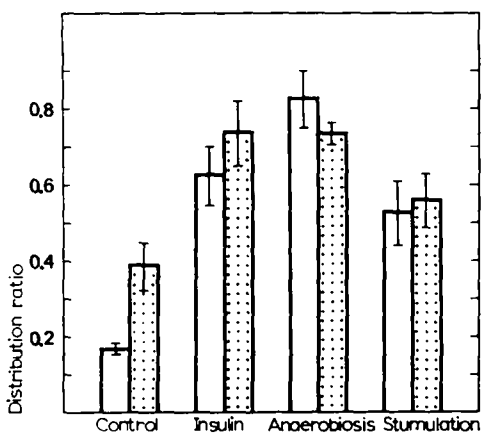


Fig. 3. Effect of preincubation *in vitro* for 30 min in the presence of insulin, anaerobiosis or stimulation on uptake *in vitro* of [$^{14}C_6$]xylose in levator ani muscle of rats treated (■) or untreated with testosterone propionate. Incubation in Krebs–Ringer bicarbonate medium containing pyruvate (10 mM) (insulin and stimulation) or glucose (11 mM) (anaerobiosis) and in the presence of [$^{14}C_6$]xylose (0.25 μC/ml) and unlabeled xylose (2 mM) lasted 30 min at 37°. The mean values of at least 3 cases are given. Vertical bars represent 2 × S.E.

of the transport kinetics shows that increased transport rates are attributable to enhanced v_{max} values, unaccompanied by changes in K_m (Fig. 2).

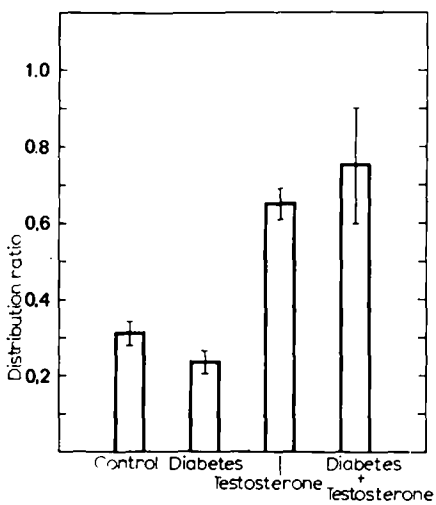
The addition *in vitro* of insulin, anaerobiosis and direct stimulation increases transport rates, direct stimulation having the least affect (Table III). The previous administration of testosterone *in vivo* does not further enhance penetration of xylose.

The presence of cyanide also stimulates sugar transport *in vitro*; here again testosterone *in vivo* is devoid of cumulative effect (Table III).

Comparison between the effects of insulin, anaerobiosis and direct stimulation —either during preincubation (Fig. 3) or at the exact start of the study on the penetration rate of xylose (Table III)—suggests that the response to each of these stimuli is subject to a different time-lag.

As the effect of testosterone on glycogen synthesis[7] and on transport of both amino acids[6] and sugars[5] shows a time-lag of about 6 h and as male hormone added *in vitro* does not influence muscle transport systems[6], the possibility that the effect of testosterone on xylose transport might be dependent on other endocrine secretions or on innervation was also tested.

Diabetes slightly but significantly decreases penetration rates of xylose in levator ani muscle (Fig. 4). This finding agrees with observations on perfused rat heart[15]; in contrast, in the preparation of intact diaphragm no decrease of the penetration rate of xylose, but only of that of 2-deoxyglucose, was observed[16]. However, the effect of testosterone is similar in both normal and diabetic rats.



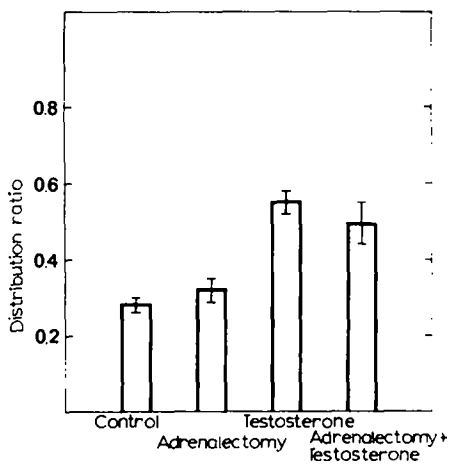


Fig. 4. Effect of alloxan diabetes on xylose uptake *in vitro* in levator ani muscle of rats treated or untreated with testosterone propionate. Experiments were performed in Krebs–Ringer bicarbonate medium (see Fig. 1) under aerobiosis at 37° an lasted 40 min. Means of at least 4 cases. Vertical bars represent 2 × S.E. Analysis of variance (F test) shows that diabetes and testosterone effects are significant ($P<0.05$ and $P<0.01$, respectively), whereas interaction among treatments is not significant.

Fig. 5. Effect of adrenalectomy on xylose uptake *in vitro* in levator ani muscle of rats treated or untreated with testosterone propionate. Experiments were performed as described (see Fig. 4). Means of 5 cases. Vertical bars represent 2 × S.E. Analysis of variance (F test) shows that testosterone effect is significant ($P<0.01$), whereas adrenalectomy effect, and interaction among treatments are not significant.

Similarly action of testosterone on sugar transport is not affected by adrenalectomy (Fig. 5) nor by hypophysectomy (Fig. 6). On the contrary acute denervation completely blocks the increase of xylose transport due to administration of male

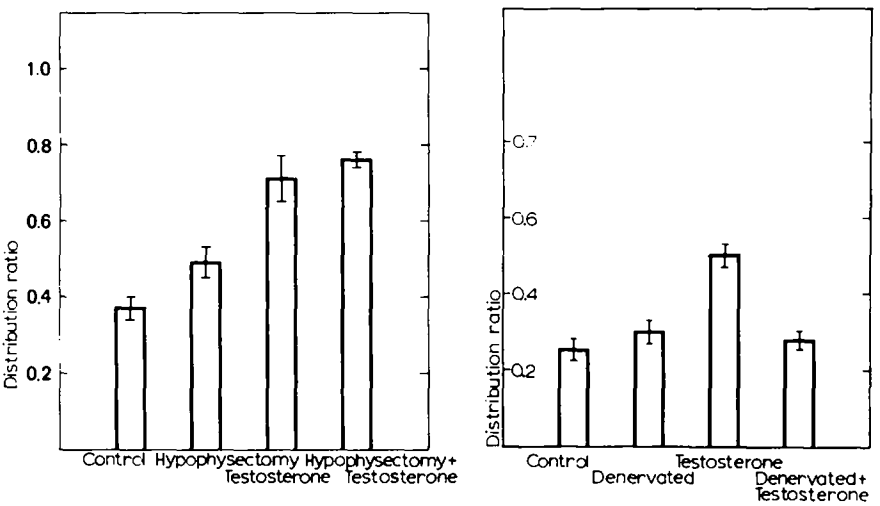


Fig. 6. Effect of hypophysectomy on xylose uptake *in vitro* in levator ani muscle of rats treated or untreated with testosterone propionate. Experiments were performed as described (see Fig. 4). Means of 5 cases. Vertical bars represent S.E. Analysis of variance (F test) shows that testosterone effect is significant ($P<0.01$), whereas the effect of hypophysectomy and interaction among treatments are not significant.

Fig. 7. Effect of denervation on xylose uptake *in vitro* in levator ani muscle of rats treated or untreated with testosterone propionate. Experiments were performed as described (see Fig. 4). Means of 11 cases. Vertical bars represent 2 × S.E. Analysis of variance (F test) shows that testosterone effect is significant ($P<0.01$), whereas denervation effect is not. Interaction between treatments is significant also ($P<0.01$).

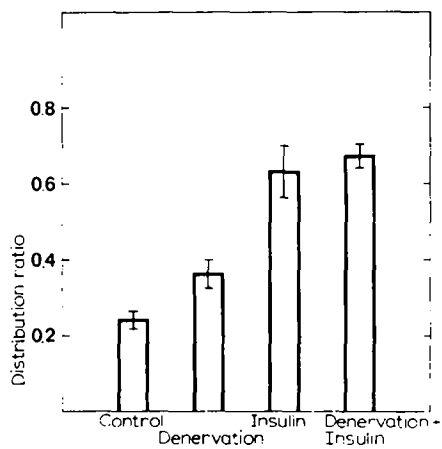


Fig. 8. Effect of insulin administration *in vivo* (1 I.U. intravenously and 1 I.U. intraperitoneally 1 h before sacrifice) on xylose uptake *in vitro* in levator ani muscle of normal and denervated rats. Animals were also given 100 mg glucose intraperitoneally. Experiments were performed as described (see Fig. 4). Means of 5 cases. Vertical bars represent 2 × S.E. Whereas insulin effect was highly significant ($P<0.01$) denervation effect and interaction between treatments were not.

hormone (Fig. 7). This is not due to nonspecific damage of transport mechanisms as the penetration rate of xylose is not modified by denervation (Figs. 7 and 8), and denervated muscle is still able to respond both to insulin or to direct stimulation *in vitro* (unpublished) and to insulin administration *in vivo* (Fig. 8).

DISCUSSION

In line with earlier reports[5–8], our results show that administration of testosterone propionate *in vivo* is followed by increased penetration of sugar into rat levator ani muscle. This increase is to be attributed to the stimulation of specific transport mechanisms, since (i) L-glucose (but see ref. 17) is unresponsive to the hormone; (ii) other sugars inhibit and phlorizin completely inhibits the observed increase; (iii) the presence of saturable processes is kinetically expressed in unchanged carrier K_m and doubled transport v_{max} values.

Finding (iii) suggests that there is an increase in the number of sites available for sugar transport. There are at least 3 possibilities: (i) the formation of new sites; (ii) the activation of sites that do not function in basal conditions; (iii) increased carrier "turnover number". The experimental data are not sufficient to eliminate any of these possibilities. It is to be remembered, however, that carriers are proteins[2,18] and that the action of testosterone is not cumulative with that of conditions which increase sugar uptake, such as the presence of insulin[19], direct stimulation[20], anaerobiosis[21,22] or cell poisons[21]. Of these, insulin[23] and stimulation or anoxia (unpublished data) show mechanisms independent of protein synthesis. This finding suggests that synthesis *de novo* of carrier molecules is not necessary for the action of the male hormone.

In this respect, it may be noted that in the case of sugar transport in the skeletal muscle and in the heart, additivity is generally found when each of the enhancing stimuli is applied at a sub-maximal level (*cf.* ref. 3); nonadditivity, on the other hand, is a general feature of maximal applications. Findings similar to those of the present paper have been reported for stimulation, insulin and anaerobiosis[24,25] or epinephrine and insulin[26] in the case of frog muscle and for testosterone and insulin in that of the levator ani[8]. ARVILL, however, has observed additivity between maximal doses of insulin and direct stimulation in the latter muscle[11].

Muscle responsiveness to sugar transport-enhancing stimuli is apparently independent of their nature and is constantly attended by increased v_{max} values (*cf.* refs. 24, 27, 28). It is therefore probable that response is in each case the result of gradual and progressive carrier activation, given the frequently proportional relationship between stimulus intensity and muscle response (*cf.* refs. 24, 27, 29), maximum response corresponding to activation of all available sites.

Yet whereas muscle response is identical under each of the experimental conditions, differences in the time-lag required before enhancement of transport begins point to clear differences in their mechanism of action on the muscle fiber. These have been shown in the case of frog muscle[24]. The action of testosterone would seem to be further distinguishable by reason of the fact that it is apparently incapable of producing maximal transport rate.

Differences in mechanisms are not apparent, however, in terms of differences in normalization after the removal of the stimulus. This is a very slow process and

begins 6 h after the suspension of direct stimulation in the case of frog muscle[24]; in that of the levator ani, no sign of normalization was observed after 4 h of incubation (unpublished data).

Carrier activation mechanisms are still not clearly understood. There can be no doubt that the majority of the experimented sugar transport-enhancing stimuli exert an influence on energy metabolism. Yet many of the reported results (*cf. e.g.* refs. 24, 29) cannot be interpreted in terms of the regulation by intracellular levels of high-energy phosphorylated compounds[21]. In the case of this muscle, depression of energy consumption induced by preincubation with 0.1 mM ouabain has no effect on xylose transport enhanced by anaerobiosis (unpublished data). It has, furthermore, been recently shown that preincubation with ouabain concentrations which block ion transport leads to increased sugar transport into the diaphragm (ref. 28). It may be noted too that testosterone itself, while it stimulates transport rates of sugar, is without meaningful effect on adenylic nucleotides and phosphocreatine levels or on ^{32}P uptake by these compounds (R. PAGNI, personal communication) in the levator ani.

Another point has to be discussed here concerning the influence of the nervous system on the effect of testosterone on sugar transport in the levator ani muscle. It is of interest to note that functional stimulation shows effects similar to those of an administration of testosterone in regard to some steps of glycogen metabolism (see ref. 7) and that levator ani muscle, being a part of the sexual apparatus of the male rat, might be influenced *via* central nervous system by the male hormone if the data of HART[30] are taken into account. Since denervation completely inhibits the effect of testosterone on muscle sugar transport, we may argue that functional or "trophic" activity of the nervous system (i) mediates the effect of testosterone or (ii) shows a "permissive" effect on male hormone action on levator ani muscle. This finding can explain the observed lack of action of testosterone applied *in vitro*[6]. As KARE *et al.*[31] report that in levator ani denervation prevails on castration also as far as muscle weight and acid-soluble P is concerned, and as the effects of direct stimulation and of testosterone on 2-deoxyglucose phosphorylation *in vitro* are not additive[32], the present result points out the need of further investigations on the relationships between testosterone and nerve effects on this muscle. This problem may be also of pharmacological interest as the levator ani test is widely employed in evaluating anabolic or "myotrophic" *versus* sexual activity of androgens.

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