Complete quantification of the total concentration of rat skeletal-muscle $\text{Na}^+ + \text{K}^+$ -dependent ATPase by measurements of [^3H]ouabain binding

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In the standard [^3H]ouabain-binding assay for quantification of the Na,K -ATPase ($\text{Na}^+ + \text{K}^+$ -dependent ATPase) concentration in rat skeletal muscles, samples are incubated for 2×60 min in $1 \mu\text{M}$ -[^3H]ouabain at 37°C followed by a wash-out for 4×30 min at 0°C . To obtain accurate determinations, values determined by this standard assay should be corrected for (1) non-specific uptake and retention of [^3H]ouabain (11% overestimation), (2) loss of specifically bound [^3H]ouabain during wash-out (21% underestimation), (3) evaporation from muscle samples during weighing (4% overestimation), (4) impurity of [^3H]ouabain (5% underestimation) and (5) incomplete saturation of [^3H]ouabain binding sites (6% underestimation). Thus corrected the standard [^3H]ouabain-binding assay determines the total Na,K -ATPase concentration. Hence, in the soleus muscle of 12-week-old rats the total [^3H]ouabain-binding-site concentration is 278 ± 20 pmol/g wet wt. This is at variance with the evaluation of the Na,K -ATPase concentration from Na,K -ATPase activity measurements in muscle membrane fractions, where the recovery of Na,K -ATPase is only 2–18%. Quantification of the total Na,K -ATPase concentration is of particular importance since it is a prerequisite for the discussion of quantitative aspects of the Na,K -ATPase.

INTRODUCTION

Several studies have documented that ouabain and other cardiac glycosides bind specifically to the Na,K -ATPase by an ATP-supported process stimulated by Na^+ and inhibited by K^+ (Albers *et al.*, 1968; Matsui & Schwartz, 1968), that this binding is saturable, reversible and stoichiometric (Hansen, 1971; Jørgensen & Skou, 1971) and that it takes place on the outside of the cell membrane (Caldwell & Keynes, 1959).

On the basis of these observations it became possible to quantify the Na,K -ATPase concentration by means of radioactively labelled digitalis glycosides, and a method for the determination of the [^3H]ouabain-binding-site concentration in small intact rat skeletal muscles *in vitro* was developed (Clausen & Hansen, 1974). Because the binding of digitalis glycosides to the Na,K -ATPase requires ATP or other phosphorylating substrates at high concentration, these measurements had to be performed on muscles where an intact metabolism could be maintained during incubations. Measurements of the [^3H]ouabain-binding-site concentration *in vivo* following intraperitoneal injection of [^3H]ouabain in rats confirmed the values obtained *in vitro* (Clausen *et al.*, 1982).

Vanadate at low concentrations was found to stabilize the Na,K -ATPase in a conformation in which digitalis glycosides can be bound independently of the presence of ATP (Hansen, 1979). This observation allowed the development of a method for the determination of the [^3H]ouabain-binding-site concentration in small muscle biopsies of around 5 mg wet wt., i.e. in samples without normal metabolism and ATP content. This method gave values for the [^3H]ouabain-binding-site concentration in rat muscles in close agreement with those obtained for whole muscles, *in vitro* or *in vivo* (Nørgaard *et al.*, 1983).

The use of vanadate allowed the determination of the [^3H]ouabain-binding-site concentration in samples of large skeletal muscles (Nørgaard *et al.*, 1983, 1984, 1985) as well as in heart muscle (Kjeldsen *et al.*, 1985; Nørgaard *et al.*, 1986).

The purpose of the present study was to identify and quantify possible sources of error in the standard [^3H]ouabain-binding assay for rat skeletal-muscle samples. This involves the evaluation of (1) the non-specific uptake and retention of [^3H]ouabain during incubations, (2) the loss of specifically bound [^3H]ouabain during wash-out of extracellular [^3H]ouabain in the cold, (3) evaporation from muscle samples during determination of the wet weight, (4) the purity of the [^3H]ouabain and (5) the degree of saturation of [^3H]ouabain-binding sites obtained after incubation. At variance with the Na,K -ATPase measurements on purified muscle membrane fractions (Jones & Besch, 1984; Clausen, 1986), the [^3H]ouabain-binding-site determinations allow corrections for the errors of the method, i.e. for incomplete quantification of the $\text{Na}^+ + \text{K}^+$ -pump concentration. This is of particular importance because complete recovery of $\text{Na}^+ + \text{K}^+$ pumps is a prerequisite for the discussion of quantitative aspects of the Na,K -ATPase.

METHODS

Animals

The experiments were carried out with 12-week-old fed female Wistar rats.

Muscle samples

After the animals had been killed by decapitation, muscles were exposed and samples with a wet weight in

the range 4–8 mg were cut out. Muscle samples were either immediately taken for measurements or kept frozen until use, as this does not interfere with the vanadate-facilitated [^3H]ouabain binding (Nørgaard *et al.*, 1983, 1984).

The [^3H]ouabain-binding-site concentration in skeletal muscles was determined with the muscle wet weight as reference. To study the evaporation associated with the determination of the wet weight, this value was followed on a Kahn microbalance for up to 10 min after exposure of the fresh muscle or after incubated biopsies had been blotted. After being weighed samples were taken for determination of the ^3H radioactivity by using liquid-scintillation counting.

[^3H]Ouabain binding

These measurements were carried out with the vanadate-facilitated (Hansen, 1979) technique (Nørgaard *et al.*, 1983, 1984). All muscle preparations were incubated in a vanadate/Tris buffer containing 10 mM-Tris chloride, 3 mM-MgSO₄, 1 mM-Tris vanadate and 250 mM-sucrose. pH was adjusted to 7.3 with Tris chloride. During incubations samples were kept agitated by continuous gassing with air. Samples were first pre-washed for 2×10 min at 0 °C.

In the standard experiments with skeletal-muscle samples, specimens were equilibrated for 2×60 min at 37 °C in buffer containing 2.08 μCi of [^3H]ouabain/ml and unlabelled ouabain added to a final concentration of 1 μM . This was followed by a wash-out in unlabelled buffer for 4×30 min at 0 °C.

The non-specific uptake of [^3H]ouabain during incubations and retention thereof after the wash-out was evaluated by incubating muscles for 2×60 min at 37 °C in buffer containing 2.08 μCi of [^3H]ouabain/ml and ouabain added to a final concentration of 0.1, 1 or 5 mM followed by wash-out for 4×30 min at 0 °C. The non-specific uptake and retention of [^3H]ouabain were compared with the uptake and retention of [^{14}C]sucrose following incubation with 1 mM-[^{14}C]sucrose and 1 mM-ouabain (Clausen & Hansen, 1974).

The methodological error associated with the wash-out in the cold was evaluated in separate wash-out experiments. After incubation for 2×60 min at 37 °C at a [^3H]ouabain concentration of 1 μM (20 $\mu\text{Ci}/\text{ml}$) the wash-out was continued for 12×30 min. The amount of ^3H radioactivity remaining in the muscle samples was determined, and by adding successively the amount of [^3H]ouabain released into the wash-out media, the ^3H radioactivity remaining in the muscles at each transfer was calculated and expressed as percentage of the initial value.

The purity of the [^3H]ouabain was determined by the Na,K-ATPase extraction method as the percentage of ^3H radioactivity that could bind to purified Na,K-ATPase (Hansen & Skou, 1973).

To determine the degree of saturation of [^3H]ouabain-binding sites obtained in the standard assay, [^3H]ouabain binding was determined in incubations of biopsies for 4×60 min at 37 °C in buffer containing [^3H]ouabain (0.20–2.08 $\mu\text{Ci}/\text{ml}$) and ouabain added to final concentrations of 0.01–5.0 μM . It was earlier shown that equilibrium is achieved after incubation for 2×60 min and 4×60 min at ouabain concentrations of 1 μM and 0.01 μM respectively (Kjeldsen *et al.*, 1986).

In standard experiments muscle samples were, after

wash-out, blotted, weighed, and soaked overnight in minivials containing 0.5 ml of 5% (w/v) trichloroacetic acid. Thereafter, 2.5 ml of a scintillation mixture (Optifluor) was added and the samples were assayed by liquid-scintillation counting of the ^3H radioactivity. On the basis of the muscle wet weight, the ^3H radioactivity in the incubation medium and the ^3H radioactivity retained in the muscle samples, the ^3H radioactivity taken up was calculated and expressed as the relative uptake, i.e. ^3H radioactivity taken up per g wet wt. of muscle, and given as decimal fraction (ml/g) or percentage filling (%). The concentration of ^3H radioactivity in the muscles was then expressed as pmol/g wet wt. by multiplying the relative uptake of ^3H radioactivity by the concentration of ouabain in the incubation medium. Thus, because standard measurements were carried out in incubations at a ouabain concentration of 1 μM , i.e. at 1000 pmol of ouabain/ml of incubation medium, the ^3H radioactivity in the muscles could be calculated and given in pmol/g wet wt. by multiplying the relative uptake of ^3H radioactivity (ml/g) by 1000 pmol/ml. To determine the total concentration of [^3H]ouabain-binding sites in muscles, the methodological errors should be corrected for.

Statistics

Results are given as means $\pm$ S.E.M. with the numbers of observations in parentheses. Statistical significance of differences was ascertained by using the two-tailed *t* test for non-paired observations.

RESULTS

The uptake and retention of [^3H]ouabain at concentrations of 0.1, 1 and 5 mM in the incubation medium was 3.3 ± 0.3 (5), 2.7 ± 0.3 (5) and $3.4 \pm 0.3\%$ filling (5) respectively. These values are not significantly different. The uptake and retention of [^3H]ouabain in incubations containing 0.1–5 mM-[^3H]ouabain is predominantly non-specific, and does not reflect a major specific binding. This is shown by the good agreement between the uptake and retention of ^3H radioactivity following incubation of muscles in 0.1 mM-, 1 mM- and 5 mM-[^3H]ouabain, and the uptake and retention of ^{14}C radioactivity following incubation in 1 mM-[^{14}C]sucrose and 1 mM-ouabain ($3.1 \pm 0.3\%$ filling). The observation that no systematic variation in the [^3H]ouabain uptake and retention is seen in the [^3H]ouabain concentration range 0.1–5 mM argues against the possibility that the subtraction of a constant value for non-specific uptake and retention of [^3H]ouabain can hide a pool of [^3H]ouabain-binding sites with low affinity (Mendel & Mendel, 1985).

For routine evaluation of non-specific uptake and retention of [^3H]ouabain, a [^3H]ouabain concentration of 1 mM in the incubation medium was used. A correction was made by subtracting the non-specific uptake and retention from the value for relative total uptake and retention determined under corresponding incubation conditions. Omission of the correction for non-specific uptake and retention results in an overestimation of the [^3H]ouabain-binding-site concentration by 11%.

Because the standard method for measuring [^3H]ouabain-binding-site concentration includes a wash-out to remove ^3H radioactivity from the extracellular space in order to increase the precision of the method, the loss of specifically bound [^3H]ouabain during this period was

Fig. 1. The wash-out of ^3H radioactivity from soleus muscle samples from 12-week-old rats

^3H]Ouabain binding was determined by the standard assay, except that samples were loaded in buffer containing $20 \mu\text{Ci}$ of ^3H]ouabain/ml and ouabain was added to a final concentration of $1 \mu\text{M}$. This was followed by wash-out for $12 \times 30 \text{ min}$ at 0°C in non-radioactive buffer. The straight line was constructed by linear-regression analysis by using the values obtained during the late slow phase of wash-out. Each point represents the mean observation on five animals, with bars denoting $2 \times \text{S.E.M.}$

investigated. This was done in separate experiments with $12 \times 30 \text{ min}$ wash-out periods with unlabelled buffer at 0°C . The ^3H radioactivity remaining in the muscles at each transfer was calculated and expressed as percentage of the initial value. These relative values were then given in a semi-logarithmic plot (Fig. 1). After about 2 h of wash-out the disappearance curve shows a linear course, indicating one remaining component. Assuming that this last component represents a monoexponential decay, the half-life or decay constant ($t_{1/2}$) was calculated. The loss of specifically bound ^3H]ouabain during the standard wash-out of $4 \times 30 \text{ min}$ was determined on the basis of the $t_{1/2}$ as the relative decrease in the fraction of ^3H radioactivity retained from 0 to 120 min. In the soleus muscle samples from 12-week-old rats $t_{1/2}$ was 7.3 h. Thus the correction factor for loss of specifically bound ^3H]ouabain during the cold wash for $4 \times 30 \text{ min}$ was calculated to be 1.21.

Because the wet weight of the muscle samples is used as reference for the ^3H]ouabain-binding-site concentration, the possible source of error associated therewith was evaluated. Groups of five muscle samples in the wet-weight range 4–7 mg were prepared from soleus muscles of 12-week-old rats. The first group of muscle samples was immediately taken for weighing, whereas the second group was incubated in vanadate/Tris buffer for the standard incubation time ($2 \times 10 \text{ min} + 2 \times 60 \text{ min} + 4 \times 30 \text{ min}$) and blotted before being weighed. When the wet weights of samples were plotted in a semi-logarithmic plot as a function of time after exposure or blotting, linear curves were obtained (Fig. 2). Thus the decrease in wet weight due to evaporation could be evaluated by using linear-regression analysis on these

logarithmically transformed values. The weight of the samples at the time when evaporation began was calculated as the intercept of the regression lines with the ordinate. Mean values were $5.7 \pm 0.4 \text{ mg}$ (5) and $4.9 \pm 0.4 \text{ mg}$ (5) for incubated and non-incubated biopsies respectively. That the regression lines are almost parallel indicates that evaporation proceeds with almost the same velocity in incubated and non-incubated biopsies as well as in small and large biopsies within the weight range studied. The wet weight of samples was decreased to 50% after 26–29 min. On this basis it was calculated that determination of sample wet weight 1 or 2 min after blotting causes an underestimation of the wet weight by 3 or 5% respectively, and thus gives an overestimation of the ^3H]ouabain-binding-site concentration by the same percentages. To ensure comparability among the weights determined in the standard assay, weighing was carried out within the second minute after blotting and with the same delay in all samples, and the error was corrected for by dividing the ^3H]ouabain-binding-site concentration by 1.04.

The ^3H]ouabain was found to have a purity of 95%. As the remaining ^3H radioactivity was assumed not to bind to the ^3H]ouabain receptor whereas it is included when the ^3H radioactivity of the incubation medium is determined, it was corrected for by dividing by a factor of 0.95.

Because the isotherm of the ^3H]ouabain binding showed a linear distribution in a Scatchard-type plot (Fig. 3), indicating a simple bimolecular reaction, the apparent dissociation constant, K_D ($0.06 \mu\text{M}$), and the maximal specific binding of ^3H]ouabain, EO_{max} .

Fig. 2. Effect of evaporation on the wet weight of muscle biopsies

Samples were prepared from soleus muscles of a 12-week-old rat and either immediately taken for weighing or incubated in vanadate/Tris buffer for $2 \times 10 \text{ min}$ at 0°C , $2 \times 60 \text{ min}$ at 37°C ($2.08 \mu\text{Ci}$ of ^3H]ouabain/ml and ouabain added to a final concentration of $1 \mu\text{M}$) and $4 \times 30 \text{ min}$ at 0°C , blotted and then taken for weighing on a Kahn microbalance. O, Biopsies dissected out and weighed; x, biopsies dissected out, incubated and weighed. Lines are constructed by using linear-regression analysis.

Fig. 3. Effect of the concentration of ouabain on the binding of [³H]ouabain in soleus muscle biopsies obtained from 12-week-old Wistar rats

The [³H]ouabain binding was measured by using the standard vanadate-facilitated method (Nørgaard *et al.*, 1983) except that incubation took place at 0.01–5 μ M-ouabain (0.20–2.08 μ Ci of [³H]ouabain/ml) for 4 $\times$ 60 min. Bound [³H]ouabain (EO) was plotted as a function of bound/free (EO/O_f) [³H]ouabain in the incubation medium. The isotherm of this Scatchard-type plot was constructed by using linear-regression analysis based upon unweighted values. After subtraction of non-specific uptake and retention, the [³H]ouabain binding was corrected for loss of specifically bound [³H]ouabain during the wash-out in the cold, evaporation during weighing and radiopurity of the [³H]ouabain. Each point represents the mean of observations on five animals with bars denoting 2 $\times$ S.E.M.

(279 pmol/g wet wt.), were determined on the basis of linear regression analysis as the slope of the line and the intercept with the ordinate respectively. Assuming the binding model is $E + O_f \rightleftharpoons EO$, in which E means receptor sites not occupied by ouabain, O_f free ouabain and EO the enzyme-ouabain complex, it was calculated that $EO = -K_D \times (EO/O_f) + EO_{max}$. The correction factor for measurements at 1 μ M-ouabain was determined to be 1.06, thus allowing for incomplete saturation of sites.

In the standard [³H]ouabain-binding assay, where muscle biopsies were incubated for 2 $\times$ 60 min in 1 μ M-[³H]ouabain at 37 °C followed by wash-out for 4 $\times$ 30 min at 0 °C, a total relative [³H]ouabain uptake of $24.1 \pm 1.5\%$ filling ($n = 5$) was found in soleus muscles from 12-week-old rats. On the basis of the present evaluation of the methodological errors associated with the [³H]ouabain-binding-site measurements, the total [³H]-ouabain-binding-site concentration was determined to be 278 ± 20 pmol/g wet wt. (5). This was calculated as $(24.1 \pm 1.5 - 2.7 \pm 0.3) \times 10$ multiplied by a correction factor of $(1.06 \times 1.21)/(0.95 \times 1.04)$. The agreement between the total specific [³H]ouabain-binding-site concentration determined on the basis of the standard assay and the Scatchard-type analysis (Fig. 3) shows high

precision and reproducibility of the [³H]ouabain-binding measurements.

DISCUSSION

In Table 1 the present value for the [³H]ouabain-binding-site concentration in soleus muscles from 12-week-old rats of 278 pmol/g wet wt. can be compared with measurements of [³H]ouabain binding in various mammalian skeletal muscles, K⁺-dependent 3-O-methylfluorescein phosphatase activity in crude muscle homogenates and Na,K-ATPase activity in purified muscle preparations. Determinations of Na,K-ATPase activity in skeletal muscles are, owing to the high background activity of non-specific ATPases, carried out on purified preparations with low recovery of Na,K-ATPase. Only in a few of such studies is the yield of plasma membrane given, allowing an estimate of the recovery of Na,K-ATPase. Such estimates are given in the lower panel of Table 1. From the measured Na,K-ATPase activity and the yield of plasma-membrane protein the Na,K-ATPase activity per g wet wt. of muscle was calculated. This value was then expressed relative to values given in Table 1 for the Na,K-ATPase activity calculated on the basis of the [³H]ouabain-binding measurements in intact muscle samples or measurements of 3-O-methylfluorescein phosphatase activity in crude muscle homogenates.

The measured [³H]ouabain-binding-site concentration varies between 100 and 800 pmol/g wet wt. The [³H]ouabain-binding-site concentration determined in the present study (278 pmol/g wet wt.) corresponds well with the earlier published value (330 pmol/g wet wt.) (Kjeldsen *et al.*, 1984). As the earlier study was performed on muscle strips incubated in Krebs-Ringer bicarbonate buffer to maintain adequate metabolism, this indicates good correlation between the earlier method (Clausen & Hansen, 1974) and the present vanadate-facilitated assay for the determination of [³H]ouabain-binding-site concentration in muscle biopsies (Nørgaard *et al.*, 1983). Generally, values around 300 pmol/g wet wt. are obtained with muscles from mature rats, young guinea pigs and human subjects. However, the muscles of 4-week-old rats have a 2.5-fold and those of adult mouse a 2-fold higher [³H]ouabain-binding-site concentration. The [³H]ouabain-binding-site concentration in rat soleus muscle is 20–30% higher than in the gastrocnemius muscle.

The measurements of the [³H]ouabain-binding-site concentration in gastrocnemius muscles from young and adult rats are in agreement with measurements of the 3-O-methylfluorescein phosphatase activity in crude muscle homogenates from the same muscle (Table 1). It should be noted, however, that the use of 3-O-methylfluorescein phosphatase measurements to determine the Na⁺ + K⁺-pump concentration is not entirely ouabain-independent, because ouabain is used for the determination of the molecular activity, and that this value is determined in partially purified enzyme preparations, whereas the activities are determined in crude preparations.

From the lower panel of Table 1 it can be seen that the recovery of Na,K-ATPase in measurements on purified membrane preparations is in the range 2–18%. Thus it is impossible to ascertain whether the enzyme activity measured is representative for the total Na,K-ATPase concentration. Large contributions from some sources of

Table 1. Measured [³H]ouabain-binding-site concentration, K⁺-dependent 3-O-methylfluorescein phosphatase activity and Na,K-ATPase activity in mammalian skeletal muscle

Values for [³H]ouabain-binding-site concentrations were converted into Na,K-ATPase activity by using a molecular activity of the Na,K-ATPase at 37 °C of 8000 cycles/min. The 3-O-methylfluorescein phosphatase (3-O-MFPase) activity was converted into Na,K-ATPase activity by using this value and a molecular activity of the 3-O-methylfluorescein phosphatase of 620 cycles/min. Na,K-ATPase activities given as $\mu\text{mol/h}$ per mg of protein were multiplied by the yield of membrane protein (mg/g wet wt.) and expressed as $\mu\text{mol/h}$ per g wet wt. The recovery of Na,K-ATPase was then calculated by dividing these values by the Na,K-ATPase activity determined in the gastrocnemius muscle from female Wistar rats by using [³H]ouabain-binding and 3-O-methylfluorescein phosphatase measurements. The values for estimated recovery of Na,K-ATPase in muscles from young rats (about 6 weeks old) given in the lower panel were calculated on the basis of the observation that the Na,K-ATPase concentration in skeletal muscle at the sixth week of life is about two-thirds of that at the fourth week (Kjeldsen *et al.*, 1984). Thus an Na,K-ATPase activity of 175 $\mu\text{mol/h}$ per g wet wt. was used [$\frac{2}{3}(245-269)$ $\mu\text{mol/h}$ per g wet wt.] for young rats. For hind-limb muscle obtained from adult rats a value of 125 $\mu\text{mol/h}$ per g wet wt. was used (121–130 $\mu\text{mol/h}$ per g wet wt.).

Species and muscle	Measured [³ H]ouabain-binding-site concentration (pmol/g wet wt.)	Calculated Na,K-ATPase activity ($\mu\text{mol/h}$ per g wet wt.)	Reference
4-week-old-rat, soleus	721	346	Clausen & Hansen (1974)
4-week-old rat, soleus	800	384	Kjeldsen <i>et al.</i> (1984)
4-week-old rat, gastrocnemius	560	269	Kjeldsen <i>et al.</i> (1984)
12-week-old rat, soleus	330	158	Kjeldsen <i>et al.</i> (1984)
12-week-old rat, gastrocnemius	270	130	Kjeldsen <i>et al.</i> (1984)
Adult mouse, soleus	620	298	Biron <i>et al.</i> (1979)
Adult mouse, soleus	670	322	Clausen & Hansen (1982)
Adult mouse, soleus	650	312	Kjeldsen <i>et al.</i> (1984)
Adult mouse, soleus	693	333	Abdel-Aziz <i>et al.</i> (1986)
4-week-old guinea pig, soleus	300	144	Kjeldsen <i>et al.</i> (1984)
4-week-old guinea pig, soleus	289	139	Kjeldsen <i>et al.</i> (1985)
Guinea pig, soleus	100	48	Brown <i>et al.</i> (1986)
Adult human, vastus lateralis	280	134	Nørgaard <i>et al.</i> (1984)
Adult human, intercostal	250	120	Nørgaard <i>et al.</i> (1984)

Species and muscle	Measured 3-O-MFPase activity ($\mu\text{mol/h}$ per g wet wt.)	Calculated Na,K-ATPase activity ($\mu\text{mol/h}$ per g wet wt.)	References
4-week-old rat, gastrocnemius	19.0	245	Kjeldsen <i>et al.</i> (1984)
12-week old rat, gastrocnemius	9.4	121	Kjeldsen <i>et al.</i> (1984)

Species and muscle	Yield of membrane protein (mg/g wet wt.)	Measured Na,K-ATPase ($\mu\text{mol/h}$ per g wet wt.)	Estimated recovery (%)	Reference
Young rat, hind limb	5.00	20.0	11.4	Rogus <i>et al.</i> (1969)
Young rat, hind limb	1.91	25.0	14.3	Asano <i>et al.</i> (1976)
Adult rat, hind limb	0.20	5.2	4.2	Andrew & Appel (1973)
Adult rat, hind limb	0.16	2.4	1.9	Kidwai <i>et al.</i> (1973)
Adult rat, hind limb	0.43	2.6	2.1	Schapira <i>et al.</i> (1974)
Adult rat, hind limb	2.50	23.0	18.4	Festoff <i>et al.</i> (1977)

enzyme activity may thus lead to misinterpretations of phenomena studied. This is evident from the difference in the effect of chemical sympathectomy when determined by using [³H]ouabain-binding assay on purified muscle homogenates (Sharma *et al.*, 1977; Sharma & Banerjee, 1978) and [³H]ouabain-binding measurements in intact rat skeletal muscle (Clausen *et al.*, 1981). The decrease obtained in the two studies was 95% and 8% respectively, indicating that the purification procedure had preferentially isolated synaptosomes. It

should be noted that the recovery problem is of general interest in the quantification of various cell components in muscles. It has been recognized to be a problem in the quantification of β -adrenoceptors (Baker *et al.*, 1980) as well as insulin receptors (Whitcomb *et al.*, 1985).

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