J. Physiol. (1982), **333**, *pp.* 367–381
With 4 text-figures
Printed in Great Britain

EFFECT OF AGE, POTASSIUM DEPLETION AND DENERVATION ON SPECIFIC DISPLACEABLE [^{3}H]OUABAIN BINDING IN RAT SKELETAL MUSCLE *IN VIVO*

BY TORBEN CLAUSEN, OTTO HANSEN, KELD KJELDSEN AND AAGE NØRGAARD

From the Institute of Physiology, University of Aarhus, 8000 Aarhus C, Denmark

(*Received 3 March 1982*)

SUMMARY

1. Following intraperitoneal injection of [^{3}H]ouabain in rats, the isotope is rapidly distributed in blood plasma available for binding to the Na–K-ATPase in the plasma membranes of most tissues. In skeletal muscle tissue excised and washed 4 × 30 min in ice-cold buffer, 95 % of the ^{3}H activity retained was shown to be [^{3}H]ouabain using a specific binding assay.

2. The [^{3}H]ouabain bound to soleus and extensor digitorum longus (e.d.l.) muscles *in vivo* and retained following wash-out in the cold showed the same saturation characteristics as those determined when binding took place *in vitro*.

3. In soleus and e.d.l. muscles obtained from 28-day-old rats, the number of [^{3}H]ouabain binding sites measured *in vivo* was 583 ± 19 and 720 ± 22 pmol/g wet wt., respectively, i.e. in good agreement with previous and present results obtained *in vitro*.

4. *In vivo* measurements showed that 7 days after denervation, the number of [^{3}H]ouabain binding sites in soleus and e.d.l. muscles was reduced by 22 and 13 %, respectively.

5. In the age interval from 28 to 85 days, the number of [^{3}H]ouabain binding sites in soleus was found to decrease by 58 %. Following I.P. injection of [^{3}H]ouabain, the 85-day-old rats showed a more pronounced and sustained rise in plasma ^{3}H activity, which in part can be due to the reduced capacity for [^{3}H]ouabain binding in skeletal muscle.

6. K depletion induced by the administration of K-deficient diet for 3 weeks reduced [^{3}H]ouabain binding by 63 % in soleus muscles. In the K-depleted animals, the plasma ^{3}H activity measured 15 min after I.P. injection of [^{3}H]ouabain was 77 % higher than in controls receiving the same dose per kg body weight.

7. The present *in vivo* results provide further support for the idea that increased digitalis toxicity due to increasing age or K depletion is related to reduced binding capacity for digitalis glycosides in skeletal muscle.

INTRODUCTION

Earlier investigations have demonstrated a specific reversible binding of [^{3}H]ouabain to the purified (Na^+–K^+)-activated ATPase, and over a wide range of enzyme

activities, there was a stoichiometric relationship between the [^{3}H]ouabain binding capacity and the ATP-splitting activity (Hansen, 1971; Jørgensen & Skou, 1971).

Digitalis glycosides are known to bind to the external surface of the plasma membrane (Caldwell & Keynes, 1959) and it was shown that measurements of the displaceable binding of [^{3}H]ouabain could be used to quantify the number of Na–K-ATPase units also in intact cells (Baker & Willis, 1972). We have developed *in vitro* techniques allowing the determination of the number of [^{3}H]ouabain binding sites in skeletal muscle, adipocytes (Clausen & Hansen, 1974, 1977) and lymphocytes (Clausen, Wang, Ørskov & Kristensen, 1980). This suggested the possibility of measuring [^{3}H]ouabain binding to tissues in the intact animal, an approach which could provide information about changes in the synthesis or degradation of Na–K pumps under *in vivo* conditions. With an intact circulation the binding of ouabain is less dependent on diffusion, allowing for rapid equilibration between the plasma and the cell surface, even in large muscles. In order to facilitate comparison with earlier data and results obtained using a wide range of sizes of animals and tissues, rats were chosen. It should be noted that the affinity of rat muscles to ouabain is relatively high. Thus, a K_D of $2{\cdot}1 \times 10^{-7}$ M was measured (Clausen & Hansen, 1974). This is in good agreement with the value of $1{\cdot}8 \times 10^{-7}$ M obtained by Erdmann, Philipp & Tanner (1976), and lower than the recently reported value of $5{\cdot}5 \times 10^{-7}$ M for human muscle (Desnuelle, Lombet, Serratrice & Lazdunski, 1982).

Here we describe a simple method for the quantification of the total number of [^{3}H]ouabain binding sites in the skeletal muscles of the intact rat. The *in vivo* results are in close agreement with those obtained *in vitro* and demonstrate the same effects of K depletion, age and denervation (see Nørgaard, Kjeldsen & Clausen, 1981; Kjeldsen, Nørgaard & Clausen, 1982*a*; Clausen, Sellin & Thesleff, 1981*b*).

METHODS

All experiments were performed using fed female Wistar rats in the age range 28–85 days (65–200 g body weight). K deficiency was induced by maintaining the animals on Altromin® pellets containing 0·75 mmol K per kg and distilled water. Metal frames were positioned in the bottom of the cages so as to prevent the animals from having access to urine or faeces. Denervation of soleus and extensor digitorum longus (e.d.l.) muscles was performed at the sciatic level.

Measurements of [^{3}H]ouabain binding in vivo and in vitro

The rats were injected intraperitoneally with a 154 mM-NaCl solution containing [^{3}H]ouabain at the indicated concentrations. In order to avoid damage or perforation of intra-abdominal organs, a blunted 0·6 or 0·8 mm injection needle was used, and all injections were given in the right side of the abdomen at the umbilical level so as to allow the solution to become distributed in the abdominal cavity above the mesenteric radix with ready access to the diaphragm. After 15–120 min the animals were killed by decapitation. Blood samples were collected from the neck vessels into glass tubes wet with a heparin solution (30 i.u./ml) and plasma obtained by centrifugation. In some experiments, the animals were anaesthetized by an intraperitoneal injection of amobarbitone. Thereafter, [^{3}H]ouabain was injected into the jugular vein and blood samples collected from the contralateral carotid artery.

Soleus and e.d.l. muscles were dissected out as previously described (Kohn & Clausen, 1971; Chinet, Clausen & Girardier, 1977). In the experiments with 28-day-old rats, whole muscles were used, whereas in the larger animals the lateral segments of soleus were carefully dissected out and used (Crettaz, Prentki, Zaninetti & Jeanrenaud, 1980). In order to minimize the dissociation of [^{3}H]ouabain from the plasma membrane, all dissections were performed as early as possible and

under continued wash with ice-cold saline. The tissues were immediately transferred into ice-cold K-free Krebs-Ringer bicarbonate buffer containing 1·27 mM-Ca and 5 mM-D-glucose. All samples from one animal were contained in a polythene cylinder closed with a nylon net at the bottom and attached to a gas inlet allowing continuous gassing with a mixture of 95% O_2 and 5% CO_2. After washing four times for 30 min in four successive tubes containing 3 ml. buffer at 0 °C, the tissues were blotted on dry filter paper mounted on a flat metal container which was kept ice-cold by circulating water from the ice bath. After weighing, the tissues were homogenized in 2 ml. 5% trichloroacetic acid, and by centrifugation a clear supernatant was obtained for the determination of ^3H activity by liquid scintillation counting. In each experiment, the specific activity of [^3H]ouabain in the solution injected was determined, and on this basis the ^3H activity retained in the tissue following the cold wash could be expressed as pmol [^3H]ouabain per g wet weight. From each animal, samples of plasma were precipitated with 5% trichloroacetic acid, and after centrifugation the ^3H activity in the clear supernatant was determined using the same counting conditions as for the tissue extracts.

For comparison, [^3H]ouabain binding to muscles was also measured *in vitro* using methods described in detail elsewhere (Clausen & Hansen, 1974, 1977).

Characterization of ^3H activity in samples of tissue and plasma by means of Na–K-ATPase

^3H activity in plasma and muscle tissue was characterized by its affinity to Na–K-ATPase according to an established procedure used for determination of the purity of [^3H]ouabain (Hansen & Skou, 1973).

Soleus, e.d.l. or hind-limb muscles were washed four times for 30 min in 3 ml. K-free Krebs-Ringer at 0 °C. They were then homogenized in 10 mM-Tris buffer (pH 7·25 at 37 °C). The homogenate (tissue/buffer ratio: 1/10) was heated for 60 min at 55 °C and the ^3H activity released separated from cellular debris by centrifugation at 100,000 *g* for 20 min. The ^3H activity of the supernatants was examined for extractability by Na–K-ATPase isolated from outer medulla of pig kidney. ATPase was prepared according to the first steps in Jørgensen's procedure (1974) and SDS-activated as described earlier (Hansen, Jensen, Nørby & Ottolenghi, 1979). To an incubation medium containing 3 mM-Mg^{2+}, 3 mM-P_i, 40 mM-Tris (pH 7·25) and an adequate aliquot of the 100,000 *g* supernatant, Na–K-ATPase was added to a final concentration of 1·17 u./ml. (ouabain binding capacity 140 nM). After 120 min incubation at 37 °C, the binding of ^3H activity was determined as described elsewhere (Hansen & Skou, 1973). A second extraction was performed as follows. An ultrafiltrate of the incubation medium was supplemented with unlabelled ouabain and ligands for the promotion of ouabain binding so as to reach the same final concentrations as in the first extraction. Finally, fresh enzyme was added and binding of ^3H activity determined once more. In control experiments [^3H]ouabain was added to muscle homogenate from animals not treated with ouabain and the extractability by Na–K-ATPase determined as described above.

A similar extraction of ^3H activity from plasma was carried out by adding plasma from [^3H]ouabain-treated animals to the incubation medium at a final concentration of 5%. Repeated extractions of ultrafiltrates were performed twice. In control experiments [^3H]ouabain was added to plasma from animals not treated with ouabain. The K concentrations were adjusted to be the same in control experiments and in the experiments with plasma or muscles from ouabain-injected animals.

Measurements of Na–K contents, ^{42}K uptake and K tolerance

In some experiments, samples of gastrocnemius muscle were taken immediately after killing, and weighed and homogenized in 5% trichloroacetic acid. After centrifugation, the Na–K contents of the clear supernatant was determined by flame photometry using an FLM photometer with internal Li standard (Radiometer, Copenhagen).

In order to determine whether the binding of ouabain to muscles *in vivo* caused the expected inhibition of active Na–K transport, the ouabain-suppressible component of ^{42}K-uptake was measured in soleus muscles obtained from rats which had been given an intraperitoneal injection of a saturating dose of ouabain 60 min before they were killed. Following preparation and a wash-out at 30 °C allowing the dissociation of around 30% of the ouabain specifically bound to the muscle cells, the uptake of ^{42}K was measured in the absence and the presence of 1 mM-ouabain. As described earlier (Clausen & Kohn, 1977), the ouabain-suppressible component of ^{42}K uptake as determined during a 20 min incubation can be taken as the Na–K pump-mediated K uptake. In order to assess

the effects of ouabain on plasma K and K tolerance, rats were anaesthetized with ketamine chloride and catheters placed in the external jugular veins on both sides. Plasma K was determined following intraperitoneal injection of ouabain, and in some instances the K tolerance was assessed by recording the changes in plasma K after an intravenous injection of a 154 mM-KCl solution (0·77 μmol/g body weight).

Isotopes, drugs and chemicals

All chemicals used were of analytical grade. K-deficient fodder was obtained from the Altromin Co, Lage, West Germany, ouabain from the Merck Co, Darmstadt, West Germany. [^{3}H]ouabain was from the New England Nuclear Corporation (Boston, U.S.) and its purity checked by the extraction method described earlier (Hansen & Skou, 1973). ^{42}K was obtained from the Danish Atomic Energy Commission, Isotope Laboratory, Risø, Denmark.

RESULTS

Identification of the ^{3}H activity in plasma and muscles following intraperitoneal injection of [^{3}H]ouabain

It is known that in rats, cardiac glycosides are metabolized and converted to other compounds (Cox, Roxburgh & Wright, 1959; Okita, 1969). Therefore, it is for the present purpose essential to identify the ^{3}H activity which is distributed in the plasma and tissues following the injection of [^{3}H]ouabain. Earlier studies have demonstrated that in isolated soleus muscles (Clausen & Hansen, 1974), as well as in frog sartorius muscle (Venosa & Horowicz, 1981), the wash-out of ^{3}H activity from pre-loaded muscles is considerably accelerated by the addition of non-labelled ouabain or digoxin to the wash-out medium. In an intact muscle, [^{3}H]ouabain released from the plasma membrane may become bound to other cell surfaces before it can gain access to the surrounding wash-out medium. Thus, the rate of ^{3}H activity release represents the net difference between the rates of over-all release and that of rebinding to vacant sites. In the presence of an excess of unlabelled ouabain or digoxin, rebinding of [^{3}H]ouabain is inhibited by competition, and considerably more [^{3}H]ouabain is readily released into the wash-out medium. Such experiments also demonstrate that the ^{3}H activity appearing in the wash-out medium, being specifically displaceable by unlabelled cardiac glycosides, is most likely to be [^{3}H]ouabain.

Sixty minutes after an intraperitoneal injection of [^{3}H]ouabain, substantial amounts of ^{3}H activity is bound in soleus and e.d.l. muscles. From Fig. 1 it can be seen that when this ^{3}H activity is washed out at 30 °C, the addition of unlabelled ouabain causes a considerable acceleration of the net release. Thus, after 180 min, only 6% of the initial tissue level was left. When the temperature is reduced to 0 °C, the fractional loss of ^{3}H activity from both soleus and e.d.l. is substantially reduced, in keeping with the well known experience that in the cold, the release of [^{3}H]ouabain from Na–K-ATPase is extremely slow (Tobin & Sen, 1970). The marked temperature sensitivity of the dissociation rate is incompatible with simple physical association and indicates that the [^{3}H]ouabain was specifically bound to the outer surface of the sarcolemma.

All the wash-out curves are closely similar to those recorded in experiments where the labelling of the muscles took place *in vitro*, again indicating that a major part of the ^{3}H activity associated with the muscles is [^{3}H]ouabain (for comparison, see Fig. 5 in Clausen & Hansen, 1974). For a further identification of the ^{3}H activity

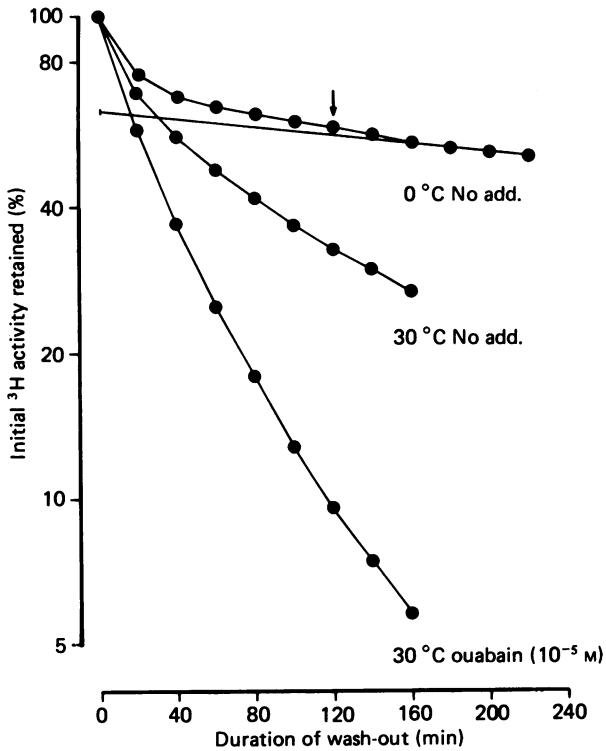

FIG. 1. The effect of temperature and unlabelled ouabain on the wash-out of ^{3}H activity from rat soleus muscles. 28-day-old rats (65 g body weight) were given an intraperitoneal injection of 0·65 ml. 154 mM-NaCl containing [^{3}H]ouabain (15 μCi/ml) and unlabelled ouabain (1·25 mM). 60 min later the animals were killed by decapitation, and soleus muscles dissected out under continued wash with ice-cold saline. The muscles were placed in polythene cylinders and transferred through a series of tubes containing 3 ml. K-free Krebs–Ringer bicarbonate buffer without or with unlabelled ouabain (10^{-5} M) and at 0 °C as indicated. At the end of wash-out, the ^{3}H activity remaining in the muscles was determined and, by adding successively the amount of ^{3}H activity released into the wash-out media, the ^{3}H activity remaining in the muscles at each transfer between the tubes was calculated and expressed as per cent of the initial level. Each curve represents the mean of two to four observations and the S.E. does not exceed the size of the symbols. The straight line was constructed by regression analysis using the values obtained during the late slow phase of wash-out at 0 °C. Its intercept with the ordinate at time zero is indicated and the small vertical bar indicates 2 × S.E. of the intercept value.

retained in the tissues, wash-out experiments were performed at 0 °C with intact soleus and e.d.l. muscles as well as segmented hind-limb muscle bundles. The ^{3}H activity recovered from these tissues after washing and homogenization in the cold was characterized by its affinity to purified Na–K-ATPase. If this ^{3}H activity is recognized as [^{3}H]ouabain by the specific binding sites of the Na–K-ATPase, a high degree of binding of the isotope can be expected at the low concentrations employed in the binding assay (Hansen, 1971). From the ^{3}H activity recovered from the tissues

TABLE 1. Extraction by Na–K-ATPase of ^3H activity from muscle homogenates obtained from [^3H]ouabain-treated rats or from homogenates to which [^3H]ouabain was added *in vitro*. 28-day-old rats were given an intraperitoneal injection of 100 μCi of [^3H]ouabain (2 nmol/g body weight). 60 min later, segments of muscles from the hind limbs or the isolated intact soleus and e.d.l. muscles were prepared and washed out for 120 min in ice-cold K-free Krebs–Ringer bicarbonate buffer containing 5 mM-D-glucose. The muscle preparations were homogenized and the homogenates heated so as to allow the release of ^3H activity bound to cellular debris. The extractability of this ^3H activity was tested using purified Na–K-ATPase and two consecutive extraction steps. Control experiments were performed using homogenates from untreated animals and determining the extractability of [^3H]ouabain added after the homogenization

Muscle preparation	Procedure	Per cent of total ^3H activity extracted by Na–K-ATPase		
		During first extraction	During second extraction	Total extractability
Hind-limb muscles Experiment 1.	Wash-out before homogenization	81·2	91·7	98·4
Hind-limb muscles Experiment 2.	Wash-out before homogenization	69·1	84·0	95·1
Hind-limb muscles Experiment 3.	Wash-out before homogenization	67·0	67·0	89·1
Soleus + e.d.l. Experiment 1.	Wash-out before homogenization	87·1	97·3	99·7
Soleus + e.d.l. Experiment 2.	Wash-out before homogenization	73·4	80·3	94·8
Hind-limb muscles	[^3H]ouabain added after homogenization	80·6	87·5	97·6

TABLE 2. Extraction by Na–K-ATPase of ^3H activity from plasma obtained from [^3H]ouabain-treated rats or from plasma to which [^3H]ouabain was added *in vitro*. 28-day-old rats were given an intraperitoneal injection of 100 μCi of [^3H]ouabain (2 nmol/g body weight). 15 or 60 min later blood samples were withdrawn and plasma isolated by centrifugation. The extractability of the ^3H activity in the plasma was tested using purified Na–K-ATPase and three consecutive extraction steps

Procedure	Per cent of total ^3H activity extracted by Na–K-ATPase			
	During first extraction	During second extraction	During third extraction	Total extractability
[^3H]ouabain 15 min *in vivo*	43·8	40·0	31·0	76·6
[^3H]ouabain 60 min *in vivo*	28·8	27·9	21·1	59·5
[^3H]ouabain 60 min *in vivo*	29·1	27·8	26·1	62·2
[^3H]ouabain added *in vitro*	52·1 ± 0·6 (3)	57·6 ± 0·8 (3)	70·9 ± 2·0 (3)	94·1 ± 0·3 (3)

and the specific activity of the injected [^{3}H]ouabain, the concentration of ouabain in the medium used for binding to Na–K-ATPase could be assumed to be in the range 10^{-9}–2×10^{-8} M i.e. considerably lower than the total binding capacity of the Na–K-ATPase added (14×10^{-8} M). From Table 1 it can be seen that for all of the above-mentioned tissues, more than 95 % of the ^{3}H activity retained after wash-out in the cold became bound to the Na–K-ATPase. This recovery is the same as that obtained when the original preparation of [^{3}H]ouabain was added directly to a homogenate of muscles from untreated animals (Table 1).

^{3}H activity in the plasma of [^{3}H]ouabain-injected animals was only partially extractable by the Na–K-ATPase, indicating that a fraction of the ^{3}H activity circulating in the intact animal is not [^{3}H]ouabain, but ^{3}H-labelled degradation products. The results presented in Table 2 indicate that 15 and 60 min after the injection of [^{3}H]ouabain, respectively 77 and 61 % of the ^{3}H activity in plasma can be identified as [^{3}H]ouabain. Control experiments, in which the original [^{3}H]ouabain preparation was added to the Na–K-ATPase showed close to 95 % extraction, indicating that the enzyme was fully capable of binding [^{3}H]ouabain even in the presence of plasma.

Although these experiments showed that a significant fraction of the ^{3}H activity in plasma was not [^{3}H]ouabain, more than 95 % of the ^{3}H activity retained by muscles after wash-out in the cold could be identified as [^{3}H]ouabain, being recognized by the highly specific binding sites on the Na–K-ATPase.

Saturation and recovery of specifically bound [^{3}H]ouabain

For a further characterization of the ^{3}H activity retained by the muscles, a series of young rats were given an intraperitoneal injection of [^{3}H]ouabain (0·15 μCi/g body weight) together with unlabelled ouabain in amounts from 0·75 to 20 nmol/g body weight, corresponding to between 1/140th and 1/5th of the LD_{50} (Lendle, 1935). From Fig. 2 it can be seen that ^{3}H activity is taken up and retained by both soleus and e.d.l. muscles, with saturation being evident at the highest doses. On the basis of the specific activity of the [^{3}H]ouabain injected, the ^{3}H activity retained in the muscles was expressed as pmol/g tissue wet wt. At the saturating dose of ouabain (12·5 nmol/g body weight), the ^{3}H activity retained by soleus and e.d.l. corresponds to 583 ± 19 and 720 ± 22 pmol/g wet wt., respectively, i.e. values closely similar to those obtained in experiments where the muscles were equilibrated with [^{3}H]ouabain *in vitro* (see Table 3 and Clausen & Hansen, 1974, 1977). Since there was no significant difference between the results obtained with 20 or 12·5 nmol/g body weight (see Fig. 2), the latter dose was chosen as appropriate for further experiments. In order to ascertain that the observed saturation reflected binding to the muscles and not variations in the absorption from the peritoneal cavity, a series of 200 g rats were injected intravenously with 1 ml. saline containing [^{3}H]ouabain (0·6–10 nmol/g body weight and 15 μCi/animal). 15 min after the injection, the animals were decapitated and the amount of ^{3}H activity retained in the muscles determined as described above. The ^{3}H activity in plasma achieved 15 min after the injection of 10 nmol/g corresponds to a ouabain concentration of $3{\cdot}5 \times 10^{-6}$ M. This is 17-fold higher than K_D for ouabain binding to muscles *in vitro*. The accumulation of ^{3}H activity showed clear evidence of saturation, attaining a plateau of around 250 pmol/g wet wt. at the

highest doses tested (5–10 nmol/g body weight). This value is closely similar to that measured using isolated soleus muscles or the intraperitoneal route of [^{3}H]ouabain administration (see Table 3).

It should be noted that even at 0 °C, a certain fraction of the [^{3}H]ouabain associated with the specific binding sites will be lost during wash-out. From the slow

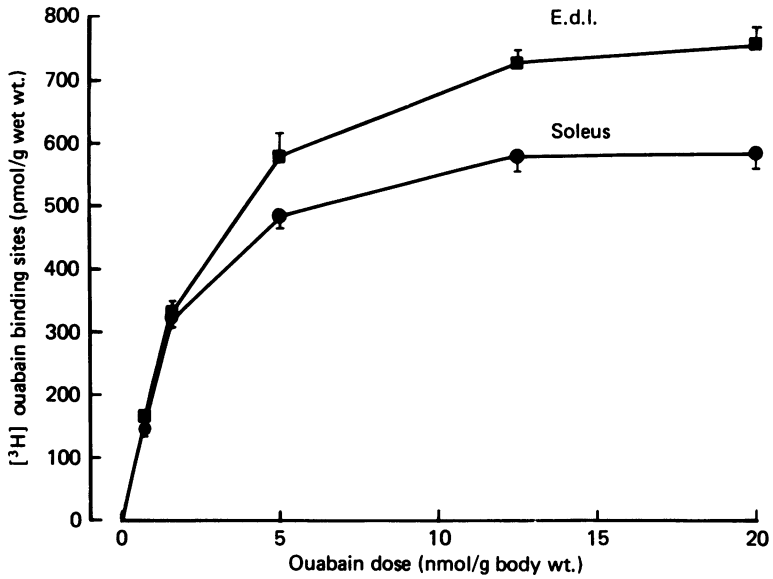

FIG. 2. Effect of ouabain dose on the binding of [^{3}H]ouabain in rat soleus and e.d.l. muscles. 28-day-old rats were given an intraperitoneal injection of 154 mM-NaCl containing [^{3}H]ouabain (15 μCi/ml) and unlabelled ouabain in the dose range from 0·75–20 nmol/g body weight. 60 min later, the animals were killed by decapitation and soleus and e.d.l. muscles were dissected out under continued wash with ice-cold saline. The muscles were washed 4 × 30 min at 0 °C in K-free Krebs–Ringer bicarbonate buffer. After blotting, weighing and homogenization in 2 ml. 5% trichloroacetic acid, the clear supernatant obtained by centrifugation was counted. On the basis of the specific activity of the [^{3}H]ouabain injected, the amount of ^{3}H activity retained in the muscles following the cold wash was expressed as pmol per g wet weight. Each point represents the mean of four to eight observations with bars denoting S.E.

component of the wash-out curve, it could be estimated that this release took place with a half-time of 11 h, i.e. somewhat slower than reported for the release of [^{3}H]ouabain from Na–K-ATPase prepared from guinea-pig hearts (half-time 9 h; see Tobin & Sen, 1970). From the straight line describing this phase of wash-out, it could be calculated that after the standard wash-out period of 120 min, around 12% of the total amount bound at time zero (read off from the intercept) was lost. On the other hand, as can be seen from Fig. 1, at 120 min (indicated by an arrow), the tissue still contained around 4% more [^{3}H]ouabain than the value read off from the straight line. This probably represents [^{3}H]ouabain still retained in the extracellular phase and reduces the total error to around 8%.

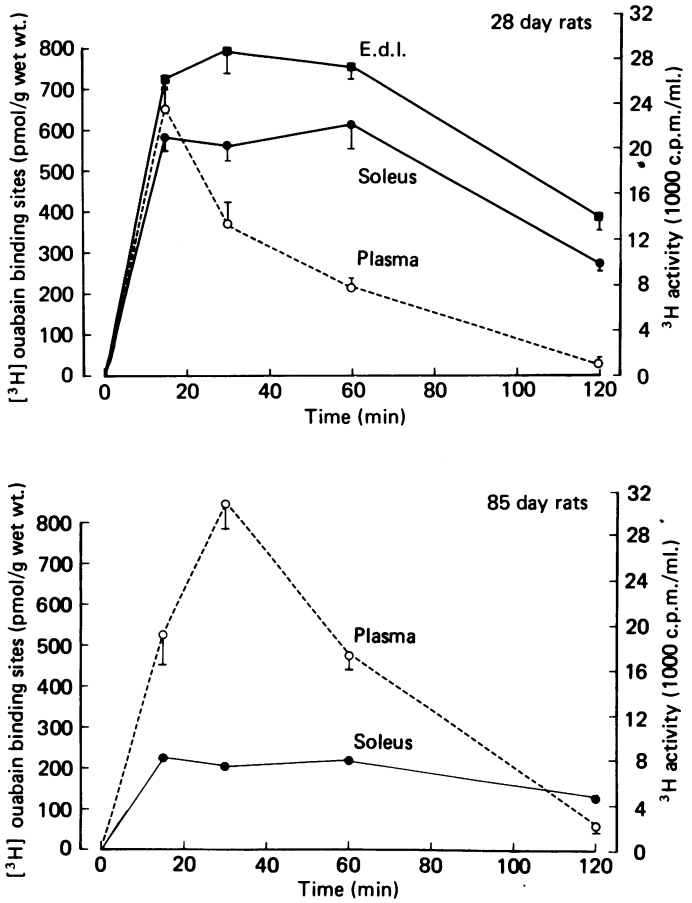

FIG. 3. Effect of age on the time course of [^{3}H]ouabain distribution in plasma, soleus and e.d.l. muscles. Rats at the ages 28 or 85 days were given an intraperitoneal injection of saline containing [^{3}H]ouabain (0·15 μCi/g body weight) and unlabelled ouabain (12·5 nmol/g body weight). 15, 30, 60 or 120 min later, the animals were killed by decapitation and blood samples collected from the neck vessels into glass tubes wet with a heparin solution (30 i.u./ml). Plasma samples obtained by centrifugation were prepared for counting of ^{3}H activity. The [^{3}H]ouabain retained by the muscles after wash-out in the cold was expressed in pmol/g wet weight. Each point represents the mean of three to eight observations, with bars denoting S.E. where this exceeds the size of the symbols.

Effects of age, K depletion and denervation on distribution of [^{3}*H*]*ouabain* in vivo

The time course of ^{3}H-activity redistribution in plasma and muscle was assessed in experiments with rats of 28 and 85 days of age. In the 28-day-old rats, the amount of [^{3}H]ouabain taken up and retained by soleus and e.d.l. rapidly increased to a plateau between 600 and 800 pmol/g wet wt., which seemed to be maintained for at least 60 min (Fig. 3, upper panel). In the 85-day-old rats, the accumulation of [^{3}H]ouabain in soleus also reached a plateau between 15 and 60 min after injection, but considerably lower values were obtained (200–250 pmol/g wet wt. (Fig. 3, lower

panel). Whereas the binding capacity for [^{3}H]ouabain in muscle decreased with the age of the animals, the rise in the ^{3}H activity of plasma was considerably larger and more sustained in the older animals (Fig. 3).

In a series of parallel experiments, the number of [^{3}H]ouabain binding sites was compared to those obtained *in vitro*. Since it has earlier been demonstrated that when measured *in vitro*, the number of [^{3}H]ouabain binding sites changes most markedly with age (Kjeldsen *et al.* 1982*a*), during K depletion (Nørgaard *et al.* 1981) and to

TABLE 3. Effect of age, denervation and K depletion on the number of [^{3}H]ouabain binding sites in soleus and e.d.l. muscles as determined *in vitro* and *in vivo*. Rats in the age range 28–85 days (65–200 g body weight) were used. The denervations were performed at the sciatic level 7 days before the experiments. K depletion was induced by the administration of a K-deficient diet for 3 weeks. The number of [^{3}H]ouabain binding sites was measured *in vitro* as earlier described (Clausen & Hansen, 1977) or *in vivo* (see legend to Fig. 2). The results are given as mean values ± S.E. with the number of observations in parentheses. The differences between results obtained *in vitro* and *in vivo* were in no instance significant ($P > 0.10$)

	[^{3}H]ouabain binding sites under saturating conditions (pmol/g wet weight)	
Age and treatment	*In vitro* results	*In vivo* results
Soleus		
28 days, untreated	584 ± 12 (4)	583 ± 19 (8)
	$P < 0.001$	$P < 0.02$
31 days, untreated	460 ± 10 (4)	495 ± 16 (6)
	$P < 0.01$	$P < 0.005$
31 days, denervated	369 ± 20 (4)	388 ± 19 (6)
35 days, untreated	433 ± 9 (4)	443 ± 9 (6)
	$P < 0.001$	$P < 0.001$
85 days, untreated	237 ± 12 (8)	247 ± 12 (12)
	$P < 0.001$	$P < 0.001$
85 days, K-depleted	95 ± 4 (8)	92 ± 11 (8)
E.d.l.		
28 days, untreated	708 ± 15 (8)	720 ± 22 (8)
	$P < 0.02$	$P < 0.02$
31 days, untreated	633 ± 15 (4)	632 ± 15 (6)
	$P < 0.30$	$P < 0.025$
31 days, denervated	602 ± 19 (4)	552 ± 24 (6)

some extent after denervation (Clausen *et al.* 1981*b*), these measurements served as a control for the accuracy of the results obtained using the *in vivo* procedure. As can be seen from Table 3, the results obtained *in vitro* and *in vivo* were in no instance significantly different, either in soleus or in e.d.l. It should be noted that after denervation the decrease in the total number of [^{3}H]ouabain binding sites, when expressed per muscle, was 56 and 39 % for soleus and e.d.l. muscles, respectively. Due to the concomitant atrophy, the number of [^{3}H]ouabain binding sites per g wet weight, however, was reduced by only 22 and 13 % for soleus and e.d.l. muscles, respectively.

Effects of the ouabain injection on plasma K, ^{42}K uptake and Na–K contents in muscle

From the experiments described above, it appeared that already 15 min after the injection of a saturating dose of [^{3}H]ouabain, virtually all Na–K pumps in skeletal muscle had bound [^{3}H]ouabain and should therefore no longer be capable of performing active Na–K transport. In keeping with this, measurements of plasma K showed that from a few minutes after the injection of ouabain, there was a progressive rise to values around twice the control level (Fig. 4). Under these

conditions, the intravenous injection of KCl (0·77 μmol/g body weight) led to cardiac arrest within 2 min. The same dose of KCl injected into control animals produced a transient rise in plasma K of only 1 mM and was well tolerated.

Gastrocnemius muscles excised 60 min after the injection of ouabain (12·5 nmol/g body weight) showed a Na gain of 35 % and a K loss of 18 % ($n = 6$, $P < 0{\cdot}001$). A more quantitative estimate of the occupancy of functional Na–K pumps by ouabain

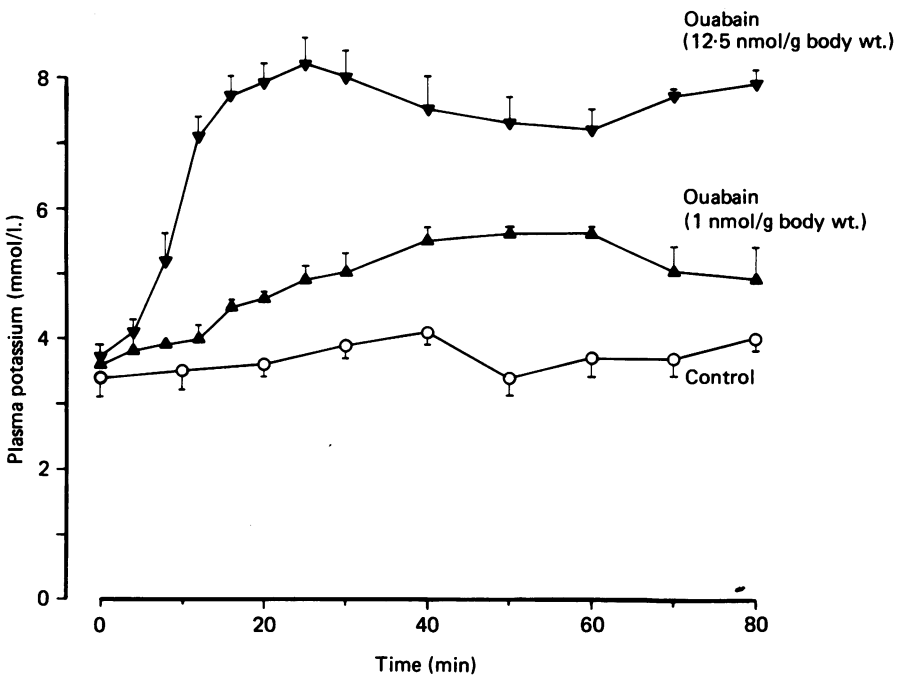

FIG. 4. Effect of ouabain on plasma potassium. 85-day-old rats were anaesthetized with ketamine chloride (initial dose 0·1 mg/g body weight). A Silastic catheter containing heparinized saline was placed in the right jugular vein for blood sampling. At time zero ouabain (1 or 12·5 nmol/g body weight) was injected intraperitoneally. Blood samples of 200 μl were withdrawn and replaced by an equivalent volume of heparinized saline. Plasma samples were obtained by 1 min centrifugation in a Beckmann microfuge, using dibutyl phthalate to allow the separation of erythrocytes from plasma. Potassium was determined by flame photometry. Each point represents the mean of four observations with bars denoting S.E.

was obtained by measuring ^{42}K uptake in soleus muscles prepared from rats pre-treated with an intraperitoneal injection of ouabain (12·5 nmol/g body weight). From Fig. 1 it can be calculated that following 70 min of wash-out at 30 °C, ouabain will be released from 30 % of the total number of binding sites, thus allowing a corresponding number of Na–K pumps to resume normal functioning. In these muscles, the ouabain-suppressible component of ^{42}K uptake was $0{\cdot}152 \pm 0{\cdot}020$ μmol/g wet weight ($n = 5$). This is 40 % of the rate measured in control muscles obtained from untreated rats. Thus, there seems to be good agreement between the [^{3}H]ouabain occupancy and the functional capacity of Na–K-ATPase in the muscles.

DISCUSSION

The major purpose of the present study has been to develop a method for the determination of specific displaceable [^{3}H]ouabain binding under *in vivo* conditions, allowing the quantification of the number of functional Na–K-ATPase units in skeletal muscle, where previous measurements have given inadequate or misleading results. Measurements of Na–K-ATPase activity in muscle have usually been performed using homogenates or membrane fractions isolated by differential centrifugation. Due to difficulties in ensuring exposure of all available [^{3}H]ouabain binding sites as well as complete recovery of Na–K-ATPase, this approach yields little or no information about the total enzyme activity per g tissue. Measurements of [^{3}H]ouabain binding to whole homogenates of mouse skeletal muscle have given values up to 128 pmol/g tissue wet weight (Lin, Romsos, Akera & Leveille, 1978). This corresponds to 19 and 27 % of the values obtained using intact mouse soleus or e.d.l. muscles (Clausen & Hansen, 1982). Measurements of [^{3}H]ouabain binding to particulate fractions obtained by differential centrifugation of muscle homogenates have suggested that sympathectomy leads to a dramatic decrease in the activity of Na–K-ATPase (Sharma & Banerjee, 1977). However, these effects could not be confirmed in measurements of [^{3}H]ouabain binding to intact muscles (Clausen, Hansen & Larsson, 1981*a*) and may be the result of preferential concentration of the sympathetic nerve endings in the particulate fractions (for a discussion of the recovery problem and its implications, see Clausen *et al.* 1981*a*, *b* and Clausen & Hansen, 1982). In view of these uncertainties, it seemed of further interest to determine the number of [^{3}H]ouabain binding sites in muscles under *in vivo* conditions, where it could be assumed that complete structural and functional integrity as well as oxygenation were preserved.

However, *in vivo* measurements are complicated by the fact that in the intact animal ouabain is to some extent metabolized (Cox *et al.* 1959; Okita, 1969). Therefore, it is essential to identify the ^{3}H activity in plasma and tissues. Using the highly specific Na–K-ATPase binding assay, it was found that 20–30 % of the ^{3}H activity in plasma was not [^{3}H]ouabain. On the other hand, when the same test was applied to the ^{3}H activity retained by the muscles following wash-out in the cold, more than 95 % could be identified as [^{3}H]ouabain. This indicates that degradation products of [^{3}H]ouabain represent an insignificant source of error. A further complication of the *in vivo* experiments is that following the injection of a saturating dose of [^{3}H]ouabain, only a certain fraction will be bound to the Na–K-ATPase in the plasma membrane, leaving appreciable amounts in the extracellular space of the muscles. Therefore, this has to be removed by a washing procedure. From Fig. 1 it can be seen that during the early rapid phase of wash-out in the cold, around one third of the total ^{3}H activity contained in the freshly prepared muscles (at the specified activity) is released. This presumably represents [^{3}H]ouabain as well as degradation products. The subsequent slow phase only approaches a straight line, probably because the [^{3}H]ouabain is released from several compartments (T-tubules, vascular tissues and the sarcolemma of deeper muscle fibres). It can be estimated that around 12 % of the specifically bound [^{3}H]ouabain is lost within the 120 min wash-out at 0 °C. As mentioned above, this error is to some extent compensated by the retention of [^{3}H]ouabain in the extracellular space, leading to a net underestimation of 8 %. Measurements performed by saturating muscles with [^{3}H]ouabain *in vitro* and

washing extracellular [^{3}H]ouabain out in the cold gave rise to a similar underestimation. At variance with the *in vitro* conditions where the [^{3}H]ouabain concentration of the buffer remains constant, the plasma [^{3}H]ouabain concentration *in vivo* was varying considerably with time (see Fig. 3). Therefore, a Scatchard plot of the *in vivo* data could not be constructed.

Direct comparison in parallel experiments show that there is no difference between the results obtained *in vitro* and *in vivo*, neither in control rats of varying age, nor in K-depleted or denervated animals. All the *in vitro* experiments were performed using a saturating concentration of [^{3}H]ouabain (2×10^{-6} M). From the plasma levels of ^{3}H activity (Fig. 3), it could be estimated that the plasma concentration in the *in vivo* experiments reached a maximum value of $3{\cdot}4 \times 10^{-6}$ M, i.e. a concentration which could be assumed to give virtually complete saturation. The changes induced with age or by K depletion or denervation could thus not be explained by competition with endogeneous glycoside-like compounds, nor could the parallel changes observed *in vitro*. On the other hand, it cannot be excluded that the changes observed *in vivo* as well as *in vitro* are the outcome of irreversible occupation of sites by endogeneous inhibitors.

Since the skeletal muscles contain a large pool of [^{3}H]ouabain binding sites, a decrease in their binding capacity will lead to a redistribution of injected [^{3}H]ouabain towards the extracellular space and plasma. However, compared to the total binding capacity of the skeletal muscles, the amount of [^{3}H]ouabain injected was large, and differences in [^{3}H]ouabain clearance via the liver or kidney might also have contributed to the observed difference in plasma levels. Further experiments with subsaturating doses of [^{3}H]ouabain are required to identify the role of changes in the peripheral binding capacity. The measurements of [^{3}H]ouabain binding in muscles lend support to the idea that the increase in digitalis toxicity developing with age may partly be the result of increased plasma concentration following from decreased peripheral binding capacity in skeletal muscle. In addition, the number of Na–K pumps in the heart was reported to decrease with age (Marsh, Lloyd & Taylor, 1981). Studies in normal infants have indicated that the peripheral volume of distribution for digoxin decreases with age (Wettrell & Andersson, 1977).

In the K-depleted rats, the plasma ^{3}H activity rose to a considerably higher level than in the age-matched controls. Thus, the increased digitalis toxicity seen during K depletion may again be explained partly by an increased plasma level resulting from diminished peripheral binding capacity (Nørgaard *et al.* 1981). This adds to the increased digitalis-sensitivity of the myocardium, which is known to be due to a higher affinity for glycoside binding at low K concentrations (Zeeman, Hirsch & Bellet, 1954; Kleiger, Seta, Vitale & Lown, 1966).

The measurements of [^{3}H]ouabain binding *in vivo* indicate that with the standard procedure adopted, virtually all Na–K-ATPase units are blocked, leading to a rapid loss of K from the muscles into the extracellular phase and plasma. This was associated with a decreased tolerance to intravenously administered KCl, indicating that the skeletal muscles and their capacity for performing active Na–K transport is decisive for the clearing of extraneous K loads. Experiments with K-depleted rats in which 70% of the [^{3}H]ouabain binding sites had been lost also demonstrated a deterioration of K tolerance (Kjeldsen, Nørgaard & Clausen, 1982*b*).

In the isolated soleus muscles prepared from rats pre-treated with ouabain, it was

possible to detect an impaired capacity for performing active Na–K transport. The fact that the rate of ouabain-suppressible ^{42}K uptake was 33% higher than the number of functional Na–K pumps would predict may be explained by a relatively higher intracellular Na concentration caused by the preceding exposure to ouabain *in vivo*.

We wish to thank Tove Lindahl Andersen and Marianne Stürup-Johansen for skilled technical assistance. The study was supported in part by grants from the Danish Medical Research Council and Aarhus Universitets Forskningsfond.

REFERENCES

BAKER, P. F. & WILLIS, J. S. (1972). Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol.* **224**, 441–462.

CALDWELL, P. C. & KEYNES, R. D. (1959). The effect of ouabain on the efflux of sodium from a squid giant axon. *J. Physiol.* **148**, 8–9*P*.

CHINET, A., CLAUSEN, T. & GIRARDIER, L. (1977). Microcalorimetric determination of energy expenditure due to active sodium–potassium transport in the soleus muscle and brown adipose tissue of the rat. *J. Physiol.* **265**, 43–61.

CLAUSEN, T. & HANSEN, O. (1974). Ouabain binding and Na^+–K^+-transport in rat muscle cells and adipocytes. *Biochim. biophys. Acta* **345**, 387–404.

CLAUSEN, T. & HANSEN, O. (1977). Active Na–K transport and the rate of ouabain binding. The effect of insulin and other stimuli on skeletal muscle and adipocytes. *J. Physiol.* **270**, 415–430.

CLAUSEN, T. & KOHN, P. G. (1977). The effect of insulin on the transport of sodium and potassium in rat soleus muscle. *J. Physiol.* **265**, 19–42.

CLAUSEN, T., WANG, P., ØRSKOV, H. & KRISTENSEN, O. (1980). Hyperkalemic periodic paralysis. Relationships between changes in plasma water, electrolytes, insulin and catecholamines during attacks. *Scand. J. clin. Lab. Invest.* **40**, 211–220.

CLAUSEN, T., HANSEN, O. & LARSSON, L.-I. (1981*a*). Sympathetic nerve terminal destruction has no effect on specific [^{3}H]ouabain binding to intact mouse and rat skeletal muscle. *Eur. J. Pharmacol.* **72**, 331–335.

CLAUSEN, T., SELLIN, L. C. & THESLEFF, S. (1981*b*). Quantitative changes in ouabain binding after denervation and during reinnervation of mouse skeletal muscle. *Acta physiol. scand.* **111**, 373–375.

CLAUSEN, T. & HANSEN, O. (1982). The Na^+–K^+-pump, energy metabolism, and obesity. *Biochem. biophys. Res. Commun.* **104**, 357–362.

COX, E., ROXBURGH, G. & WRIGHT, S. E. (1959). Research papers. The metabolism of ouabain in the rat. *J. Pharm. Pharmac.* **11**, 535–539.

CRETTAZ, M., PRENTKI, M., ZANINETTI, D. & JEANRENAUD, B. (1980). Insulin resistance in soleus muscle from obese Zucker rats. *Biochem. J.* **186**, 525–534.

DESNUELLE, C., LOMBET, A., SERRATRICE, G. & LAZDUNSKI, M. (1982). Sodium channel and sodium pump in normal and pathological muscles from patients with myotonic muscular dystrophy and lower motor neuron impairment. *J. clin. Invest.* **69**, 358–367.

ERDMANN, E., PHILIPP, G. & TANNER, G. (1976). Ouabain-receptor interactions in ($Na^+ + K^+$)-ATPase preparations. A contribution to the problem of nonlinear Scatchard plots. *Biochim. biophys. Acta* **455**, 287–296.

HANSEN, O. (1971). The relationship between g-strophanthin-binding capacity and ATPase activity in plasma-membrane fragments from ox brain. *Biochim. biophys. Acta* **233**, 122–132.

HANSEN, O. & SKOU, J. C. (1973). A study on the influence of the concentration of Mg^{2+}, P_i, K^+, Na^+ and Tris on ($Mg^{2+} + P_i$)-supported g-strophanthin binding to ($Na^+ + K^+$)-activated ATPase from ox brain. *Biochim. biophys. Acta* **311**, 51–66.

HANSEN, O., JENSEN, J., NØRBY, J. G. & OTTOLENGHI, P. (1979). A new proposal regarding the subunit composition of ($Na^+ + K^+$)-ATPase. *Nature, Lond.* **280**, 410–412.

JØRGENSEN, P. L. (1974). Purification and characterization of ($Na^+ + K^+$)-ATPase. III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. *Biochim. biophys. Acta* **356**, 36–52.

JØRGENSEN, P. L. & SKOU, J. C. (1971). Purification and characterization of ($Na^+ + K^+$)-ATPase. I. The influence of detergents on the activity of ($Na^+ + K^+$)-ATPase in preparations from the outer medulla of rabbit kidney. *Biochim. biophys. Acta* **233**, 366–380.

KJELDSEN, K., NØRGAARD, AA. & CLAUSEN, T. (1982*a*). Age-dependent changes in the number of ^{3}H-ouabain-binding sites in rat soleus muscle. *Biochim. biophys. Acta* **686**, 253–256.

KJELDSEN, K., NØRGAARD, AA. & CLAUSEN, T. (1982*b*). Effect of K-depletion on the Na–K pumping capacity in skeletal muscle and the K-tolerance of rats. *Eur. J. clin. Invest.*, Abstract (in the Press).

KLEIGER, R. E., SETA, K., VITALE, J. J. & LOWN, B. (1966). Effects of chronic depletion of potassium and magnesium upon the action of acetylstrophanthidin on the heart. *Am. J. Cardiol.* **17**, 520–527.

KOHN, P. G. & CLAUSEN, T. (1971). The relationship between the transport of glucose and cations across cell membranes in isolated tissues. VI. The effect of insulin, ouabain, and metabolic inhibitors on the transport of 3-O-methylglucose and glucose in rat soleus muscles. *Biochim. biophys. Acta* **225**, 277–290.

LENDLE, L. (1935). Digitaliskörper und verwandte herzwirksame Glykoside (Digitaloide). In *Handbuch der Experimentellen Pharmakologie*, 1st edn., ed. HEUBNER, W. & SCHULLER, J., p. 78. Berlin: Springer Verlag.

LIN, M. H., ROMSOS, D. R., AKERA, T. & LEVEILLE, G. A. (1978). Na^+, K^+-ATPase enzyme units in skeletal muscle from lean and obese mice. *Biochem. biophys. Res. Commun.* **80**, 398–404.

MARSH, A. J., LLOYD, B. L. & TAYLOR, R. R. (1981). Age dependence of myocardial Na^+–K^+-ATPase activity and digitalis intoxication in the dog and guinea pig. *Circulation Res.* **48**, 329–333.

NØRGAARD, AA., KJELDSEN, K. & CLAUSEN, T. (1981). Potassium depletion decreases the number of ^{3}H-ouabain binding sites and the active Na–K transport in skeletal muscle. *Nature, Lond.* **293**, 739–741.

OKITA, G. T. (1969). Distribution, disposition and excretion of digitalis glycosides. In *Digitalis*, ed. FISCH, C. & SURAWICS, B., pp. 13–26. New York: Grune & Stratton.

SHARMA, V. K. & BANERJEE, S. P. (1977). The effect of 6-hydroxydopamine on specific [^{3}H]ouabain binding to some sympathetically innervated organs of the cat. *Molec. Pharmacol.* **13**, 796–804.

TOBIN, T. & SEN, A. K. (1970). Stability and ligand sensitivity of [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. *Biochim. biophys. Acta* **198**, 120–131.

VENOSA, R. A. & HOROWICZ, P. J. (1981). Density and apparent location of the sodium pump in frog sartorius muscle. *J. Membrane Biol.* **59**, 225–232.

WETTRELL, G. & ANDERSSON, K.-E. (1977). Clinical pharmacokinetics of digoxin in infants. *Clin. Pharmacokinetics* **2**, 17–31.

ZEEMAN, S., HIRSCH, S. & BELLET, S. (1954). The effect of potassium depletion induced by desoxycorticosterone acetate on the lethal dose of lanatoside C in dogs: Relationship of plasma levels, skeletal and cardiac muscle potassium content to the lethal dose. *Am. J. med. Sci.* **277**, 65–73.