Biochimica et Biophysica Acta, 345 (1974) 387–404

BBA 76631

OUABAIN BINDING AND Na^+–K^+ TRANSPORT IN RAT MUSCLE CELLS AND ADIPOCYTES

TORBEN CLAUSEN and OTTO HANSEN

Institute of Physiology, University of Aarhus, 8000 Århus C (Denmark)

(Received November 14th, 1973)

SUMMARY

1. The accumulation and the release of [^{3}H]ouabain has been characterized in isolated intact soleus muscles and free fat cells of the rat. The number of ouabain-binding sites was determined and compared with the rates of active Na^+–K^+ transport.

2. In soleus muscle, [^{3}H]ouabain is bound to the surface of the plasma membrane by a reversible and saturable process, which is inhibited by K^+, Li^+, 2,4-dinitrophenol, the omission of Na^+ or the addition of digoxin.

3. The release of [^{3}H]ouabain from preloaded muscles is accelerated by K^+, 2,4-dinitrophenol and unlabelled ouabain or digoxin, presumably because of diminished binding of the glycoside.

4. Under steady-state conditions for ouabain binding a kinetic analysis indicates that soleus muscles contain $7.2 \cdot 10^{-10}$ moles binding sites per g wet wt or 3350 per μm^2 of sarcolemma surface area. An apparent dissociation constant of $2.1 \cdot 10^{-7}$ M was found.

5. Measurements of ^{22}Na efflux and ^{42}K influx under basal conditions indicate that the number of Na^+ and K^+ transported per ouabain-binding site correspond to respectively 500 and 325 per min.

6. In isolated fat cells ouabain binding showed qualitatively the same characteristics as in soleus muscle, although the rate was considerably faster and the affinity higher (apparent dissociation constant: $1.7 \cdot 10^{-8}$ M).

7. The fat cells were estimated to contain $2.0 \cdot 10^{-11}$ moles ouabain-binding sites per ml of cells or 66 per μm^2 of plasma-membrane surface. The ouabain-sensitive component of ^{42}K influx corresponds to 3450 ions per site per min.

8. It is concluded that in intact muscle cells and adipocytes, which constitutes the major portion of total body weight in mammals, the binding of ouabain is qualitatively closely similar to that described in microsomal ($Na^+ + K^+$)-activated ATPase.

INTRODUCTION

Cardiac glycosides specifically inhibit the active transport of Na^+ and K^+ in a wide variety of tissues [1–5], and with the observation that these compounds

prevent the activation of a membrane-bound ATPase by Na^+ and K^+ (ref. 6), they have become the tool of choice for the characterization of this enzyme and the mechanism of active Na^+–K^+ transport.

A number of studies have shown that radioactively labelled glycosides are bound to (Na^++K^+)-activated ATPase in microsomes prepared from a variety of sources [7–9] and to intact cells [10–14].

In squid axons, ouabain was only found to inhibit the active Na^+–K^+ transport when added to the external surface of the plasma membrane [15] and in "inside-out" vesicles prepared from human erythrocytes, the binding of the glycoside was only a minute fraction of the amount bound to "right-side out" vesicles [16].

However, little information is available about the uptake of ouabain in the most common cell types in mammals, i.e. skeletal muscle cells and adipocytes. The present investigation was undertaken with the purpose of characterizing the uptake of [^{3}H]ouabain in isolated soleus muscles and adipocytes of the rat and to correlate these data with the rate of Na^+–K^+ transport determined under similar conditions.

The results indicate that in these cells [^{3}H]ouabain is bound to the external surface of the plasma membrane by a specific, saturable and reversible process, which is markedly influenced by other cardiac glycosides, temperature, cellular ATP and the concentration of Na^+ and K^+ in the incubation medium.

METHODS

Experiments with soleus muscles

The procedures for the preparation and incubation of isolated soleus muscles have been described in detail elsewhere [17]. Muscles weighing 30–40 mg were obtained from fed Wistar rats in the weight range 60–70 g. All experiments were performed at 30 °C with the exception of some wash-out experiments which were done at 0 °C. The standard incubation medium was K^+-free Krebs–Ringer bicarbonate buffer [18] containing 1.27 mM Ca^{2+} and 5 mM glucose. In order to maintain the concentration of K^+ in the incubation media at a level which would not interfere significantly with the exchange of [^{3}H]ouabain, each incubation with the labelled glycoside was preceeded by a wash of 10 min duration. During the incubations with [^{3}H]ouabain a certain release of cellular K^+ could not be prevented, but by choosing a sufficiently large incubation volume, the concentration of K^+ in the medium (as checked by flame photometry at the end of incubation) did not exceed 0.8 mM.

The uptake of [^{3}H]ouabain or [^{14}C]sucrose in soleus muscles was determined after blotting on dry filter paper and homogenization in 4 ml of 5% trichloroacetic acid containing 0.1 mM unlabelled ouabain (carrier). After centrifugation, aliquots of 0.5 or 1.0 ml of the clear supernatant were withdrawn for the determination of ^{3}H radioactivity using Bray's scintillation mixture [19] or a more recently developed mixture [20] and a Packard liquid scintillation spectrometer No. 3320. The ^{3}H radioactivity of the incubation medium was determined under similar conditions, and the results corrected for quenching by external standardization.

The wash-out of [^{3}H]ouabain was assessed as previously described for other compounds [17]. After loading with the labelled glycoside the muscles were attached to polyethylene tubes and transferred through a series of incubation tubes con-

taining 3 ml of buffer without labelled ouabain. At the end of wash-out, the amount of ^{3}H radioactivity retained in each muscle was determined as described above, and the ^{3}H radioactivity released into the wash-out media was measured using 0.5-ml aliquots. After correction for volume and quenching, the amount of ^{3}H radioactivity retained by each individual muscle throughout the wash-out period and the rate coefficient of release were calculated as previously described [21]. The efflux of ^{22}Na and the influx of ^{42}K were determined at 30 °C as described elsewhere [5].

Experiments with adipocytes

Whole epididymal fat pads were prepared from fed Wistar rats weighing 100–120 g and washed in K^+-free Krebs–Ringer bicarbonate buffer containing 1% dialyzed bovine serum albumin, 5 mM D-glucose and 1.27 mM Ca. Incubation took place at 37 °C in a metabolic shaker moving 100 cycles/min, using polyethylene counting vials containing 2–6 ml buffer. The amount of [^{3}H]ouabain taken up by the fat pads was determined after homogenization in 2 ml 5% trichloroacetic acid containing 10^{-4} M ouabain as carrier. 0.5-ml aliquots were counted as described above.

Isolated fat cells were prepared essentially as described by Rodbell [22], using fat pads from rats in the same weight range as those used for the experiments with whole fat pads. After the collagenase treatment, the cells were washed 4 times in K^+-free Krebs–Ringer bicarbonate buffer containing 5 mM glucose, 1.27 mM Ca^{2+} and 1% dialyzed bovine serum albumin and suspended in the same medium. This was contained in a polyethylene vial with a magnetic stirrer and continuous gassing with a mixture of O_2 (95%) and CO_2 (5%). From this homogeneous suspension which was kept at 37 °C, aliquots were transferred to the various incubation media, using a polyethylene automatic pipette.

Incubation took place at 37 °C in a metabolic shaker moving 100 cycles/min using polyethylene vials containing 2 ml buffer.

The isolated cells were sampled by the procedure recently developed by Gliemann et al. [23]: 200-μl aliquots were transferred to polyethylene microcentrifuge tubes containing 100 μl (two drops) of dinonylphtalate. By centrifugation for 60 s at 10 000 $\times g$ in a Beckman microcentrifuge (Catalogue No. 314300) the cells were carried through the dinonylphtalate (specific gravity 0.98) and layered on the top of this as a thin disk, essentially devoid of contamination with extracellular phase. The top of the centrifuge tube containing the cells and the top of the dinonylphtalate layer was cut off and directly transferred into a counting vial containing 10 ml of a liquid scintillation mixture [20]. 50-μl aliquots of the incubation medium were counted in the same mixture, and after correction for quenching, the ^{3}H radioactivity was converted to nmoles of ouabain accumulated per ml of fat cells. The fat cell content of the incubation media was determined either by weighing the total triglyceride content after extraction and drying [24], or simply by direct measurement of the cell volume after centrifugation of the suspension in a hematocrit tube.

The ouabain-sensitive component of K^+-influx in isolated fat cells was determined by measuring the accumulation of ^{42}K and the [^{14}C]sucrose space at 37 °C with the same technique for the sampling of cells. ^{42}K radioactivity was determined by suspending the pellet of cells in 4 ml of water and measuring the Cerenkov radiation produced [25]. Following the decay of ^{42}K, the ^{14}C radioactivity was determined by liquid scintillation counting [19].

Experiments with a preparation of $(Na^+ + K^+)$-activated ATPase

Microsomal $(Na^+ + K^+)$-activated ATPase was prepared from ox brain as described by Skou and Hilberg [26]. Ouabain binding was carried out as described elsewhere [9]. The effect of cold ouabain on the release of [^{3}H]ouabain from the enzyme preparation was studied after incubation of enzyme with 3 mM Mg^{2+}, 120 mM Na^+, 3 mM ATP, 40 mM Tris (pH 7.25, 37 °C) and $5 \cdot 10^{-8}$ M [^{3}H]ouabain at 37 °C. The incubation was terminated after 60 min by placing the sample on an ice bath. The sample was then centrifuged in the cold and the pellet resuspended in buffers of the composition indicated in the legend to Fig. 11. The amount of [^{3}H]ouabain retained by the microsomes was determined by the ultrafiltration method [9] during 1 h of incubation at 37 °C.

Chemicals and isotopes

All chemicals used were of analytical grade. Bovine serum albumin was purchased from Sigma Co. (St. Louis) and used after dialysis against distilled water for 24 h at 4 °C, followed by neutralization with NaOH. Ouabain was obtained from Merck (Darmstadt) and [^{3}H]ouabain from the New England Nuclear Corporation (Boston). The specific activity was 13 Ci/mmole, and the purity of the preparations was checked by the extraction method described earlier [27]. Only solutions with purities of more than 93% were used. [U-^{14}C]sucrose (0.6 Ci/mmole), and ^{22}Na (0.1 Ci/mmole) were products of The Radiochemical Centre, Amersham. ^{42}K (0.1 Ci/mmole) was obtained from The Danish Atomic Energy Commission, Isotope Laboratory, Risø.

RESULTS

A. Soleus muscle

In order to define the conditions under which the distribution of ouabain in the muscles had reached a steady state, the effect of concentration on the time course of uptake was determined. From Fig. 1 it can be seen that at 10^{-3} M ouabain, the space occupied by the label does not differ significantly from that available to [^{14}C]-sucrose and that a constant level is reached 30 min after the onset of incubation. At 10^{-8} M the accumulation of label quickly exceeds the size of the total water space (which was found to correspond to 0.774 ml/g wet wt), and continues to increase over a period of 4 h.

A further lowering of the concentration did not lead to any increase in the initial rate of accumulation. At 10^{-9} M the space occupied by label after 60 and 240 min of incubation corresponded to 1.89 ± 0.10 and 5.21 ± 0.38 ml/g wet wt, respectively.

In the concentration range $2.5 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M, the amount of [^{3}H]ouabain taken up reached a constant level after 4 h. By keeping the isotope concentration (μCi/ml) constant at varying concentrations of ouabain and assuming that the uptake of [^{3}H]ouabain measured at 10^{-3} M merely represents equilibration with the interstitial space, the amount of [^{3}H]ouabain confined to the cells may be calculated by subtracting the space occupied at 10^{-3} M from that obtained with the lower concentrations. The inset of Fig. 2 shows that the uptake calculated in this way is saturable, and the straight line obtained by plotting the data in a Scatchard-type plot is com-

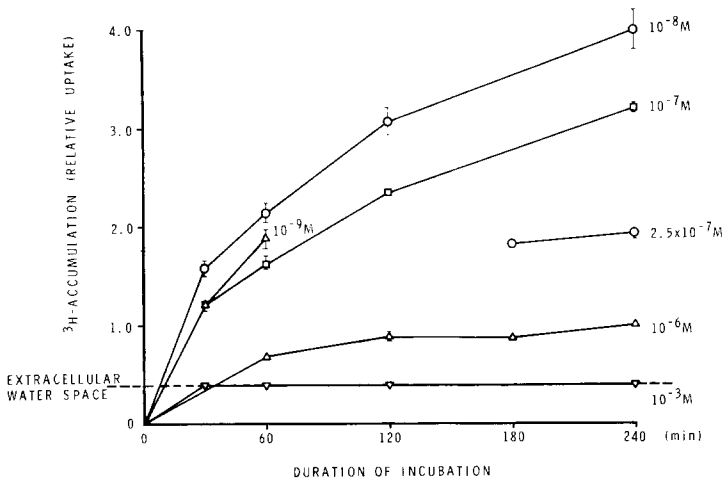

Fig. 1. Time course of ouabain accumulation in intact rat soleus muscle. Isolated rat soleus muscles were washed in K^+-free Krebs-Ringer bicarbonate buffer and incubated in 10 ml of this milieu containing 5 mM D-glucose and ^{3}H-labelled ouabain at the concentrations indicated. Incubation took place at 30 °C under continuous gassing with a mixture of 95 % O_2 and 5 % CO_2. The ^{3}H radioactivity in the muscles was determined after trichloroacetic acid extraction and in each instance compared with that of the incubation medium. The ^{3}H radioactivity accumulated per g wet wt is divided by the ^{3}H radioactivity per ml of incubation medium and expressed as a relative uptake (ml/g wet wt). Each point represents the mean of 3–4 observations with bars indicating 2 times S.E. where this exceeds the size of the symbols. The horizontal dashed line denotes the extracellular space as determined with [^{14}C]sucrose (0.392 ± 0.007; $n = 4$).

patible with the idea that the glycoside (O) is taken up into a single pool (E) by a reversible process ($E+O \rightleftharpoons EO$) [9] with an apparent dissociation constant of $2.1 \cdot 10^{-7}$ M.

The maximum uptake of ouabain calculated from a linear regression by the method of least squares is $7.21 \cdot 10^{-10}$ moles/g wet wt.

The soleus muscles were found to contain 2000 fibers, and assuming that these were of uniform cylindrical shape, the surface area of the sarcolemma was calculated to comprise $1.3 \cdot 10^{11}$ μm^2/g wet wt. Thus, an uptake of $7.2 \cdot 10^{-10}$ mole/g of muscle would correspond to $6.5 \cdot 10^9$ molecules of ouabain per muscle cell or 3350 molecules per μm^2 of sarcolemma.

Since only minute amounts of ^{3}H radioactivity were incoporated into glycogen or the trichloroacetic acid precipitate of the muscle proteins, it seems unlikely that the accumulation of ^{3}H radioactivity is due to metabolic conversion.

[^{3}H]Ouabain may either be accumulated in the cytoplasm or bound to the outer surface of the plasma membrane. If significant amounts were taken up into the cells, the disruption of their integrity would be expected to prevent an accumulation of label to a concentration exceeding that of the incubation medium. Cutting the muscles into five segments was found to induce a loss of 90% of the intracellular K^+. However, even in this preparation 60 min of incubation with 10^{-7} M of [^{3}H]ouabain led to an accumulation of label considerably exceeding the total water space and not markedly differing from the level obtained with intact muscles (Figs. 3A and B).

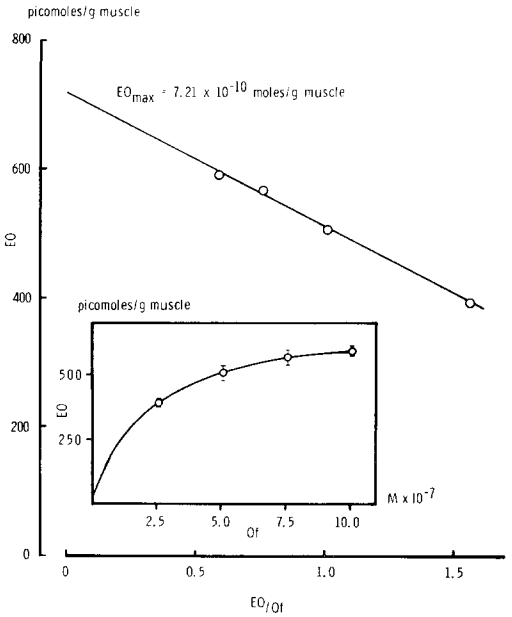

Fig. 2. Plot of "bound" (*EO*) versus "bound/free" (*EO*/*Of*) [^{3}H]ouabain in soleus muscle. Experimental conditions as in Fig. 1, except that the incubation in all instances lasted for 240 min. The inset shows *EO* (i.e. the relative uptake in ml/g wet wt at the ouabain concentration indicated less the relative uptake at a ouabain concentration of 10^{-3} M times the ouabain concentration indicated) as a function of the concentration of free ouabain (*Of*) in the incubation medium, each point representing the mean of four observations with bars denoting 2 times S.E. The regression line of the Scatchard-type plot has been constructed using the method of least squares and the calculated value for the intercept with the ordinate is indicated.

Digoxin (10^{-4} M), which has been shown to compete with ouabain for the binding sites in microsomal membranes [8], suppressed the accumulation of [^{3}H]ouabain both in the intact and in the cut soleus muscles to levels closely similar to those obtained with 10^{-3} M ouabain (Figs 3A and B).

This together with the observation that even in the "cut" muscles the accumulation of [^{3}H]ouabain is saturable (data not presented) argues that it reflects the binding of the glycoside to the outer surface of the plasma membrane (see also Fig. 7).

Binding of ouabain to a microsomal membrane preparation may be supported by ATP in combination with Na^+ and Mg^{2+} and is then inhibited by K^+, other monovalent cations and other glycosides. In order to identify the pool or site involved in the accumulation of [^{3}H]ouabain in the intact cells, these factors were investigated. In the intact muscles, the addition of ATP (0.5 mM) caused a slight stimulation of [^{3}H]ouabain accumulation, but since the same effect was obtained with EDTA, it is presumably not related to energy metabolism. However, 2,4-dinitrophenol (0.1–1.0 mM) produced a considerable suppression (Figs 3A and B), indicating that a depletion of intracellular ATP interferes with the binding of ouabain.

Fig. 4 shows the effects of Na^+, K^+, and Li^+ on the accumulation of [^{3}H]-ouabain measured after 60 min incubation at a concentration of 10^{-7} M. Partial replacement of NaCl by sucrose caused a slight increase followed by a progressive in-

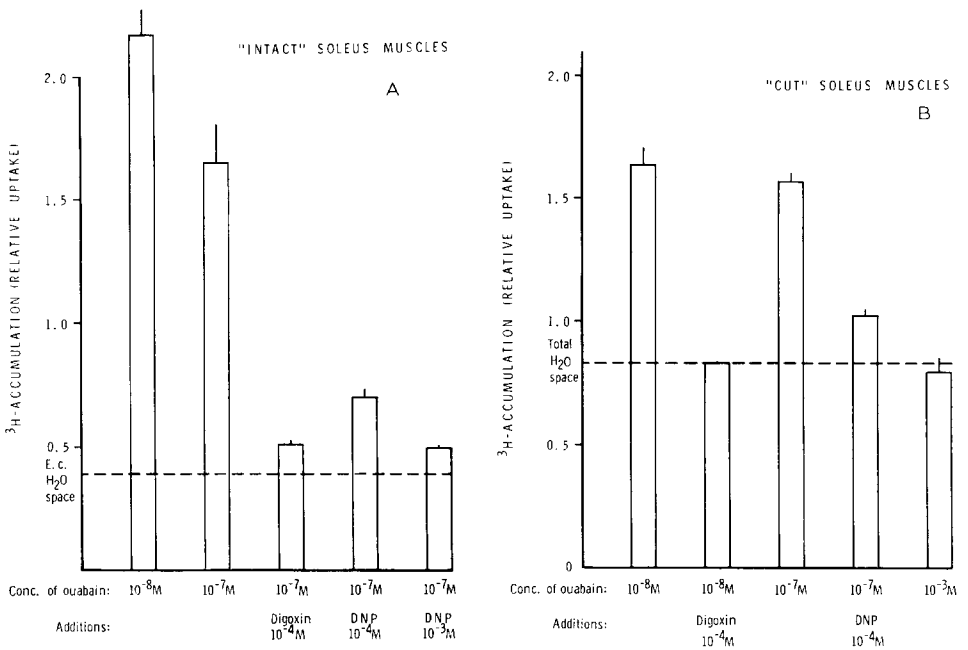

Fig. 3. (A) Effect of digoxin and 2,4-dinitrophenol on the accumulation of [^{3}H]ouabain in intact soleus muscle. Experimental conditions as described for Fig. 1, except that the incubation in all instances lasted for 60 min. Each column represents the mean of four observations with bars denoting S.E. The horizontal dashed line indicates the extracellular space as determined with [^{14}C]sucrose. (B) Effect of digoxin and 2,4-dinitrophenol on the accumulation of [^{3}H]ouabain in cut soleus muscle. Experimental conditions as described for Fig. 1, except that prior to the wash and incubation, all the muscles were cut transversely into five segments of approximately the same size. All incubations lasted for 60 min. The horizontal dashed line indicates the total water content as determined by weighing before and after drying for 2 h at 110 °C. Each column represents the mean of four observations with bars denoting S.E.

hibition at the lowest concentrations of Na^+. The inhibitory effect of Na^+ substitution seems to be most pronounced in the Tris-buffered medium, but also in the standard Krebs–Ringer bicarbonate buffer the total replacement of NaCl caused a significant suppression. Substitution with Li^+ and in particular with K^+ caused a considerably more pronounced inhibition, which is probably not solely due to Na^+ lack.

Preincubation in Na^+-free Li^+-containing Krebs–Ringer bicarbonate buffer for 120 min caused no significant change in the accumulation of [^{3}H]ouabain measured during a subsequent 30-min incubation in the standard K^+-free Krebs–Ringer bicarbonate. This indicates that the effect of Li^+ is exerted at the outer surface of the plasma membrane. The prompt effect of changes in extracellular K^+ concentration (see Fig. 6) suggests that this ion also preferentially acts at the outer surface of the plasma membrane.

The reversibility of [^{3}H]ouabain accumulation was assessed in a series of wash-out experiments. Soleus muscles were loaded for 120 min in a buffer containing 10^{-6} M [^{3}H]ouabain, i.e. conditions which would allow almost complete equilibration between the tissue and the medium (see Fig. 1). When washed in buffer contain-

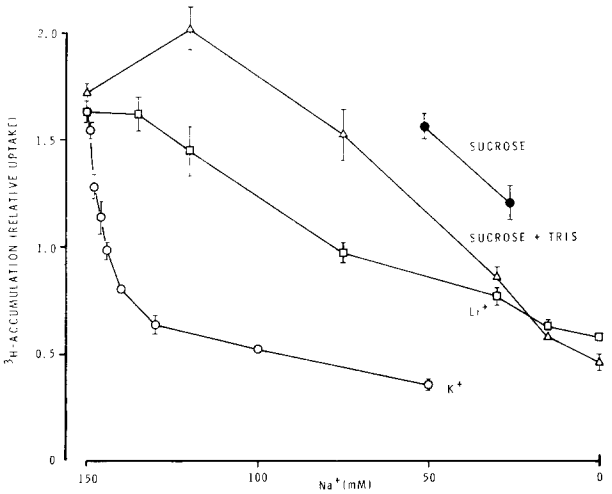

Fig. 4. Effect of Na^+, K^+, Li^+ and Tris on the accumulation of [^{3}H]ouabain in intact soleus muscle. Experimental conditions as described for Fig. 1, except that all incubations lasted for 60 min and took place in buffer containing 10^{-7} M [^{3}H]ouabain. Na^+, the only monovalent cation of the standard incubation medium was replaced by equivalent amounts of either K^+ or Li^+. For the points denoted "sucrose", NaCl was partially or totally replaced by an isoosmolar amount of sucrose, keeping the concentration of $NaHCO_3$ constant. For the curve denoted "Tris–sucrose", NaCl was partially or totally replaced by an isoosmolar amount of sucrose, $NaHCO_3$ was omitted and the solution buffered to a pH of 7.4 with Tris (10 mM). During incubation, this solution was continuously bubbled with 100% O_2. Each point represents the mean of 3–10 observations with bars denoting 2 times S.E. where this exceeds the size of the symbols.

ing no ouabain these muscles were found to release the ^{3}H radioactivity. After the initial rapid phase (which presumably represents washout of [^{3}H]ouabain from the extracellular phase) the release proceeds at a slowly decreasing rate (Fig. 5). It appears that the addition of unlabelled ouabain or digoxin to the wash-out medium produces a prompt acceleration of ^{3}H release.

The rate coefficient of ^{3}H release which represents a more sensitive parameter for the wash-out process is illustrated in Fig. 6. The addition of K^+ (6 mM) to the efflux medium produced a prompt rise, which presumably in part reflects inhibition of the reattachment of [^{3}H]ouabain already released from the plasma membrane. This interpretation is also supported by the observation that the wash-out of [^{3}H]-ouabain is inhibited when the conditions for binding are ameliorated by the omission of K^+.

The addition of 2,4-dinitrophenol (0.1 mM) increased the rate coefficient of [^{3}H]ouabain release by 72% within 30 min (data not presented).

In microsomal ATPase cooling has been found to cause a considerable decrease in the rate by which bound ouabain is released [28, 29]. From Fig. 7 it can be seen that when the wash-out of [^{3}H]ouabain was performed at 0 °C, the release proceeded at a slower rate than at 30 °C (Fig. 5). Cutting the muscles into three segments caused only a rather modest and transient rise in the release of ^{3}H radioactivity. The fact that, in spite of such severe leaks in the plasma membrane, the muscles retained the major part of their [^{3}H]ouabain, argues strongly that the glycoside is not accu-

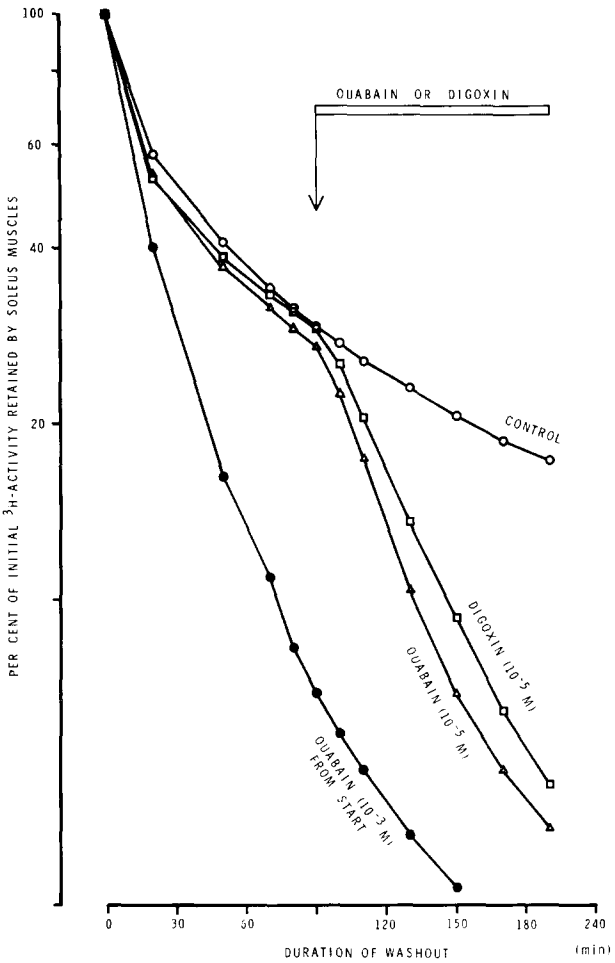

Fig. 5. The effect of ouabain and digoxin on the wash-out of [^{3}H]ouabain from soleus muscles. Soleus muscles were loaded with [^{3}H]ouabain by incubation for 120 min in K^+-free Krebs–Ringer bicarbonate buffer containing 10^{-6} M [^{3}H]ouabain (2 μCi/ml) and D-glucose (5 mM). After blotting, the muscles were mounted on polyethylene tubes and transferred through a series of tubes containing 3 ml of the standard incubation medium without or with the additions indicated. At the end of the wash-out period, the ^{3}H radioactivity remaining in the muscle was determined and by adding successively the amount of ^{3}H radioactivity released into the wash-out media, the ^{3}H radioactivity retained in the muscles at each transfer between the tubes was calculated and expressed as per cent of the initial level. In the case of the lowest curve, unlabelled ouabain (10^{-3} M) was present from the onset of wash-out; at the arrow, either ouabain (10^{-5} M) or digoxin (10^{-5} M) was added to separate groups of muscles. Each curve represents the mean of 2–4 observations.

mulated in the cytoplasm. In contrast, when the temperature of the wash-out medium was increased to 30 °C, the rate of ^{3}H release showed an immediate and marked increase.

When the wash-out was performed at 0 °C, the rate coefficient of [^{3}H]ouabain release was approximately constant from 90 min after the onset, and it seemed justified, therefore, to extrapolate the late straight portion of the efflux curve back to the

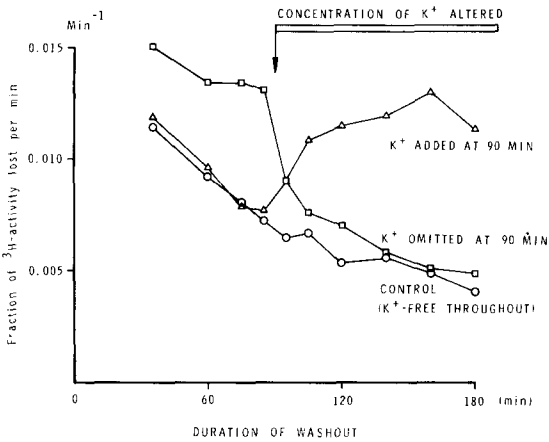

Fig. 6. The effect of K^+ on the wash-out of [^{3}H]ouabain from soleus muscles. Experimental conditions as described for Fig. 5. After the loading, the muscles were washed either in K^+-free Krebs–Ringer bicarbonate buffer throughout, ○–○; in K^+ -free buffer up till 90 min; △–△; or in buffer containing 6 mM K^+ up till 90 min, □–□. At the arrow, K^+ (6 mM) was either added or omitted from the wash-out medium as indicated. The results have been expressed as the fraction of ^{3}H radioactivity released per min [21], and each curve represents the mean of two observations.

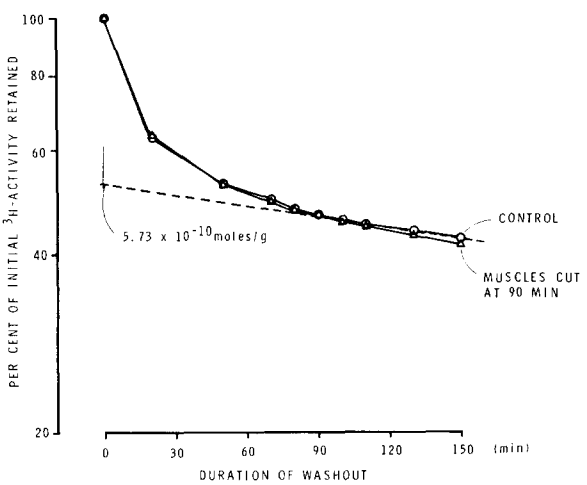

Fig. 7. The effect of tissue damage on the wash-out of [^{3}H]ouabain from soleus muscles in the cold. Experimental conditions as described for Fig. 5, with the exception that all of the wash-out took place at 0 °C, and that the muscles were transferred between the wash-out tubes using polyethylene baskets instead of the direct attachment of the achilles tendon used in the other wash-out experiments. 90 min after the onset of wash-out, all of the muscles in one group were cut transversely into three segments of approximately the same size, and the wash-out allowed to continue immediately afterwards. The dashed line indicates the extrapolation of the straight portion of the wash-out curve back to the onset of wash-out, and the value indicated at the intercept with the ordinate has been calculated on the basis of the concentration of [^{3}H]ouabain in the loading medium. Each curve represents the mean of three observations.

onset of wash-out. This gave an intercept value of $5.7 \cdot 10^{-10}$ moles/g wet wt which is essentially the same as the amount bound after 4 h of incubation at 10^{-6} M ouabain ($5.9 \cdot 10^{-10}$ moles/g wet wt.; see Fig. 2)

In order to determine the relationship between the number of binding sites and the ouabain-sensitive component of the active Na^+–K^+ transport, the rates of ^{22}Na efflux and ^{42}K influx were measured under similar conditions. The only modification was that K^+ was added to a final concentration of 5.93 mM and that glucose was not added to the incubation medium. Fig. 8 shows the effect of ouabain on the rate coefficient of ^{22}Na release from preloaded soleus muscles.

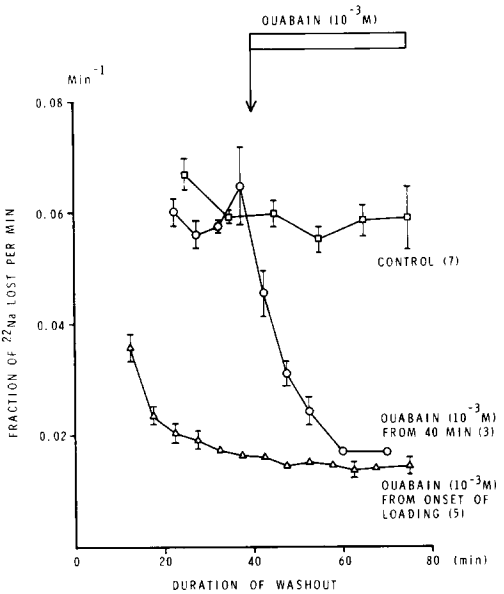

Fig. 8. The effect of ouabain on ^{22}Na efflux from rat soleus muscle. Soleus muscles were loaded for 60 min in Krebs–Ringer bicarbonate buffer containing 6 mM K^+ and ^{22}Na (10 μCi/ml) without or with ouabain (10^{-3} M). After blotting, the muscles were transferred through a series of tubes containing Krebs–Ringer bicarbonate buffer, without or with ouabain as indicated. 40 min after the onset of wash-out, ouabain (10^{-3} M) was added to the wash-out medium for one group of the muscles. Each point represents the mean of 3–7 observations, with bars denoting 2 times S.E.

At a concentration of 10^{-3} M, the glycoside caused an inhibition of 70% within 25 min. The size of the intracellular Na^+-pool was determined either by extrapolating the efflux curve back to the onset of wash-out or by measuring the amount of ^{22}Na taken up in the water space not available to [^{14}C]sucrose [5]. By multiplying these values by the decrease in rate coefficient produced by a maximal concentration of ouabain (10^{-3} M), the amount of ^{22}Na extruded per min by the ouabain-sensitive mechanism was 0.388 ± 0.077 or 0.339 ± 0.016 μmoles/g wet wt, respectively. These estimates correspond to, respectively, 1.67 and 1.45 times the ouabain-sensitive component of ^{42}K uptake determined under the same conditions (0.233 μmoles/g wet wt/min) [5]. Thus, it would appear that for each ouabain-binding site, around 500 Na^+ and 325 K^+ are transported actively per min.

B. Adipocytes

Both in the isolated fat cells and in the whole epididymal fat pads, the space available to [^{3}H]ouabain at a concentration of 10^{-3} M was not significantly different from that occupied by [^{14}C]sucrose. Because of the small size of the intracellular water space it is difficult to exclude with certainty that ouabain to some extent could gain access to the intracellular space. However, there is no indication that the glycoside is incorporated into the triglycerides.

Isolated fat cells rapidly accumulated [^{3}H]ouabain, and in the concentration range $1 \cdot 10^{-8}$–$2.5 \cdot 10^{-7}$M, a constant level was obtained within 60 min. From Fig. 9 it can be seen that the uptake is saturable and the Scatchard plot is compatible with the idea that the glycoside is accumulated in a single pool. This analysis indicates that the isolated fat cells may bind $1.6 \cdot 10^{-11}$ moles of ouabain per ml of cells. In another experiment performed under the same conditions a value of $2.5 \cdot 10^{-11}$ moles was found. The mean cell diameter was 32.6±0.9 μm ($n = 162$), and from the two determinations it could be calculated that the number of binding sites is around 65 per μm^2 of plasma-membrane surface.

The addition of K^+ (5.93 mM) to the incubation medium produced a marked inhibition of the uptake (Fig. 9), and digoxin completely displaced [^{3}H]ouabain from preloaded cells within 20 min (data not presented).

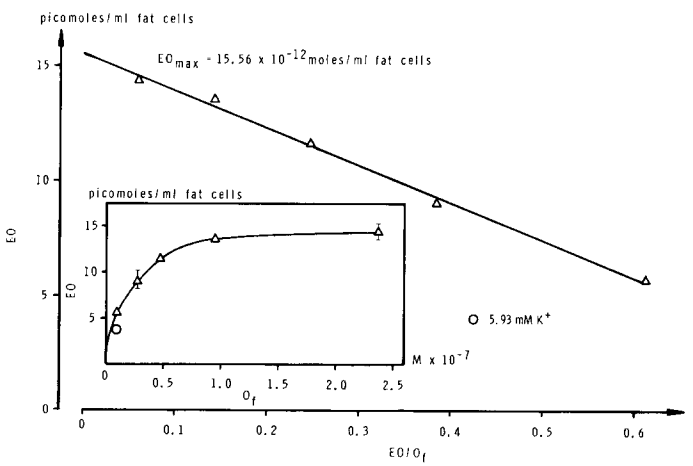

Fig. 9. Plot of "bound" (*EO*) versus "bound/free" (*EO*/*Of*) [^{3}H]ouabain in fat cells. Isolated fat cells were washed in K^+-free Krebs–Ringer bicarbonate buffer containing 1 % dialyzed bovine serum albumin and 5 mM glucose. Aliquots of the cell suspension were pipetted into polyethylene vials containing 2 ml buffer with the additions of [^{3}H]ouabain or K^+ indicated. After 60 min of incubation, triplicate samples of the cells were separated from the incubation medium and their ^{3}H radioactivity determined .The inset shows *EO* (i.e. the relative uptake in ml/g wet wt at the ouabain concentration indicated less the relative uptake at a ouabain concentration of 10^{-3} M times the ouabain concentration indicated) as a function of the concentration of free ouabain (*Of*) in the incubation medium, each point representing the mean of three observations with bars denoting 2 times S.E. The regression line of the Scatchard-type plot has been constructed using the method of least squares, and the calculated value for the intercept with the ordinate is indicated. In one instance K^+ (5.93 mM) was added to the incubation medium and the result of this experiment is denoted by ○.

In whole epididymal fat pads, the accumulation of [^{3}H]ouabain was found to proceed at a considerably slower rate. At a concentration of 10^{-7} M, a constant level of $2.8 \cdot 10^{-11}$ moles/ml was only reached after 120 min of incubation. At that concentration, the fat cells reached a plateau within 5 min.

Fig. 10 shows the time course of ^{42}K uptake in the absence and in the presence of a maximal concentration of ouabain (10^{-3} M). As assessed from the initial part of the curves, the ouabain-sensitive component of the K^+ accumulation was 69 nmoles/ml cells per min, which corresponds to 3450 K^+ per ouabain-binding site per min.

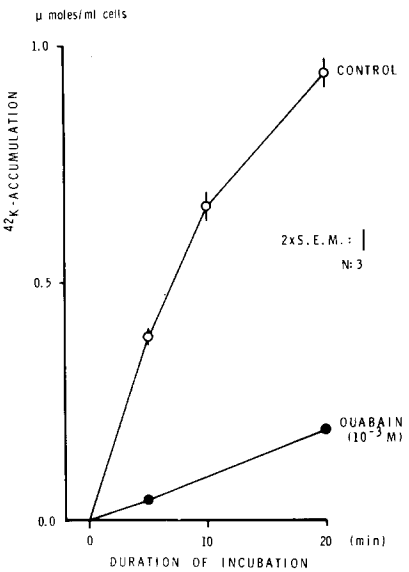

Fig. 10. The effect of ouabain on the time course of ^{42}K uptake in fat cells. Isolated fat cells were incubated in Krebs–Ringer bicarbonate buffer containing 5.93 mM K^+, 1 % albumin and 5 mM glucose with ^{42}K (1 μCi/ml) and [^{14}C]sucrose (0.5 μCi/ml). At the indicated intervals, aliquots of 200 μl were withdrawn from the incubation suspension and the cells separated for determination of ^{42}K radioactivity and ^{14}C radioactivity. The amount of ^{42}K taken up into the space not available to [^{14}C]sucrose is expressed as μmoles/ml of fat cells and the bars denote 2 times S.E.

C. Microsomal ATPase

In order to evaluate whether the inhibitory effect of 2,4-dinitrophenol on the binding of ouabain to soleus muscle could possibly be due to a direct effect rather than a lowering of the cellular ATP content, the binding of [^{3}H]ouabain to microsomal ($Na^+ + K^+$)-activated ATPase from ox brain was measured. It was found that although the omission of ATP from the incubation medium completely abolished the binding of [^{3}H]ouabain, the addition of 2,4-dinitrophenol (0.1 or 1.0 mM) in the presence of ATP produced no change whatsoever.

The decrease in the rate of [^{3}H]ouabain binding to soleus muscle induced by the omission of Na^+ might be attributed to direct effects of the substitutes per se. Whereas the addition of sucrose (250 mM) caused no significant change in the binding of [^{3}H]ouabain to the microsomal ATPase, Tris (165 mM) induced a rather marked

inhibition. This may in part account for the more pronounced inhibition of [^{3}H]ouabain binding obtained when Tris was used to replace Na^+ (Fig. 4).

The observation that the release of [^{3}H]ouabain from preloaded muscles is accelerated by the addition of unlabelled ouabain or digoxin indicates that a significant part of the labelled ouabain already dissociated can return and become bound to the tissue (see Discussion). In order to determine whether unlabelled ouabain had any effect on the dissociation of labelled ouabain from the binding sites per se, the following experiments were performed: by taking advantage of the stability of the enzyme–ouabain complex at 0 °C, the release of labelled ouabain from microsomal ATPase can be studied after separation of bound and free ouabain by centrifugation in the cold and resuspension at 37 °C [28, 29]. From Fig. 11 it can be seen that when the microsome preparation was resuspended in buffer without ATP (i.e. under conditions, where the binding of labelled ouabain already released from the binding sites is excluded), the release of [^{3}H]ouabain was not influenced by the addition of unlabelled ouabain, indicating that this process per se was not affected. Conversely, when the microsomes were resuspended in a buffer containing all the constituents required for binding (Na^+, Mg^{2+} and ATP), the net release of [^{3}H]ouabain was considerably slower. As predicted by the reversible nature of ouabain binding, the

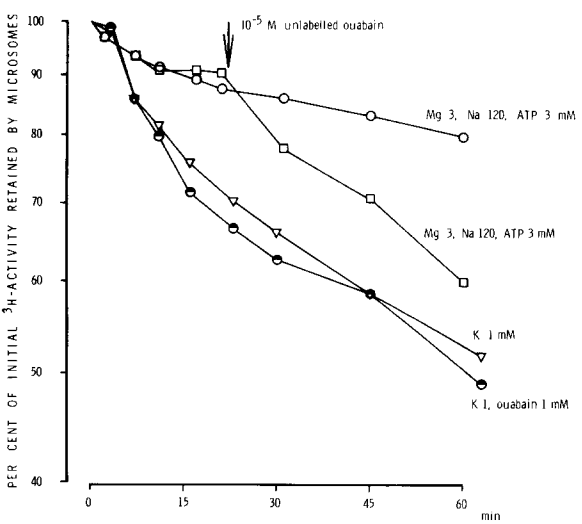

Fig. 11. The effect of unlabelled ouabain on the release of [^{3}H]ouabain from microsomal ATPase. Ox brain enzyme, 0.185 mg/ml, ouabain-sensitive activity 205 μmoles P_i/mg protein per h, was incubated with 3 mM Mg^{2+}, 120 mM Na^+, 3 mM ATP, 40 mM Tris (pH 7.25, 37 °C) and $5 \cdot 10^{-8}$ M [^{3}H]ouabain at 37 °C. The incubation was terminated after 60 min by placing the sample on an ice bath. After centrifugation at 100 000 $\times g$ for 20 min in the cold, the supernatant was discarded and the sediment washed twice with ice-cold 40 mM Tris buffer. The sediment was then resuspended in ice-cold Tris buffer (40 mM) with either 1 mM K^+, ▽–▽; 1 mM K^+ and 1 mM unlabelled ouabain, ◓–◓; or 3 mM Mg^{2+}, 120 mM Na^+ and 3 mM ATP, ○–○ or □–□, and incubated at 37 °C. After 22 min, a small volume of unlabelled ouabain was added (at the arrow) so as to give a final concentration of 10^{-5} M in one of the tubes containing Mg^{2+}-Na^+-ATP, □–□. The release of [^{3}H]ouabain at 37 °C was followed for 60 min and expressed as per cent of the amount of [^{3}H]ouabain retained at the onset of incubation at 37 °C.

addition of unlabelled ouabain promptly accelerated the rate of release to a level almost identical to that obtained in the buffer without ATP. Thus, it seems reasonable to conclude that the wash-out of [^{3}H]ouabain from the muscles represents the net result of dissociation and reassociation (between the labelled molecules and binding sites) and that the process of dissociation per se is not directly influenced by an excess of unlabelled ouabain in the extracellular phase.

DISCUSSION

The ($Na^+ + K^+$)-activated microsomal ATPase has been shown to bind ouabain by an ATP-supported process, which is stimulated by Na^+ and markedly inhibited by K^+ and by other cardiac glycosides [7, 8]. The present study has shown that intact skeletal muscle cells and adipocytes accumulate [^{3}H]ouabain in a qualitatively similar manner. For a further comparison, it is necessary to determine whether the accumulation of [^{3}H]ouabain in the intact cells reflects metabolism, transport or binding. The observation that the ^{3}H radioactivity is neither incorporated into protein, glycogen or triglycerides excludes the first possibility. Since the cutting of soleus muscles into several segments neither prevents them from accumulating ^{3}H radioactivity to a concentration exceeding that of the incubation medium, nor accelerates the release of [^{3}H]ouabain already taken up, it seems unlikely that the glycoside is accumulated inside the plasma membrane. Furthermore, the inhibitory effect of K^+ or 2,4-dinitrophenol on uptake and the prompt stimulation of release induced by the same factors cannot readily be reconciled with the idea that the glycoside penetrates the plasma membrane. Finally, the space occupied by ouabain at a high concentration (10^{-3} M) even after 4 h of incubation does not exceed that available to sucrose. It is concluded, therefore, that this represents filling of an extracellular space and that the higher values for relative uptake obtained at lower concentrations of ouabain represents a combination of filling of the extracellular space and binding to the outer surface of the plasma membrane. The last-mentioned fraction of the uptake, when converted into absolute amounts of ouabain accumulated, shows saturation, and the data are compatible with binding according to the mass-law equilibrium described for microsomal ($Na^+ + K^+$)-activated ATPase: $E + O \rightleftharpoons EO$ [9]. E is the concentration of sites available to ouabain in the intact cells. O is the concentration of free ouabain, and EO is the concentration of sites which have already bound ouabain. It should be noted that E is defined as $EO_{max} - EO$ and that the true number of sites may be higher.

In accordance with this simple model the number of available ouabain-binding sites can be determined, either by measuring the steady-state accumulation at various extracellular concentrations, or by the simpler procedure of loading the muscles at 10^{-6} M ouabain and measuring the amount released during a wash-out in the cold (Fig. 7).

An interesting question is whether the number of ouabain-binding sites determined by these methods is comparable with the values reported for muscle homogenates. In soleus muscle, the calculated number of ouabain-binding sites is $7.2 \cdot 10^{-13}$ moles/mg muscle (wet weight). Assuming a generally accepted molecular activity of around 7000 (i.e. that 7000 molecules of ATP are degraded per min per ouabain-binding site) and a protein content of 15% of the wet weight, the number of

ouabainbinding sites would correspond to a specific ($Na^+ + K^+$)-activated ATPase activity of the soleus muscle of $7.2 \cdot 10^{-13} \cdot 7000 \cdot 60 \cdot 1/0.15 = 2.0$ μmoles P_i/mg protein per h. The figures given in the literature for the ouabain-suppressible ATPase activity of whole homogenates of deoxycholate-treated calf hearts and canine hearts are 1.3–1.8 and 2.8 μmoles P_i/mg protein per h, respectively [30, 31]. Similar values for whole homogenates of skeletal muscles are not available, but partly purified ATPase preparations have been obtained from this tissue after fractional centrifugation and detergent treatment of the homogenate. From hamster skeletal muscles ATPase preparations with specific activities of 11–51 μmoles P_i/mg protein per h have been isolated [32]. From canine heart a purified ATPase preparation with specific activity as high as 200 μmoles P_i/mg protein per h was recently isolated [31].

According to the same reasoning, the number of ouabain-binding sites determined in the fat cells would correspond to a specific activity of $1/0.9375 \cdot 60 \cdot 7000 \cdot 2.0 \cdot 10^{-11}$ moles/g per min = 9.0 μmoles P_i/g per h. This is of the same order of magnitude as the value reported for the ouabain-sensitive component of ($Na^+ + K^+$)-activated ATPase found in lysates of isolated mouse fat cells (15.5 μmoles P_i/g per h) [33].

HeLa cells were reported to bind ouabain with an apparent dissociation constant of $1.4 \cdot 10^{-8}$ M (ref. 12). This is in the same range as the value found for isolated fat cells in the present study ($2 \cdot 10^{-8}$ M), and not far from the apparent dissociation constants reported for microsomal ($Na^+ + K^+$)-activated ATPase prepared from ox brain ($2 \cdot 10^{-9}$–$1 \cdot 10^{-8}$ M) [27].

On the other hand, the apparent dissociation constant calculated for soleus muscles is $2.1 \cdot 10^{-7}$, indicating that in this preparation the affinity for ouabain is at least one order of magnitude lower.

Whereas in the above-mentioned preparations the ouabain-binding sites are presumably readily accessible for the glycoside, the complex structure of a whole intact muscle implies that diffusion can be rate-limiting for the exchange of substances between the surfaces of the plasma membrane and the surrounding incubation medium. This is presumably the case for 3-*O*-methylglucose [17], and in the present study it was found that when the concentration of ouabain in the incubation medium is lowered, the initial rate of relative uptake approach a constant value (Fig. 1). The simplest explanation for this is that the outer fibres in the muscle bind ouabain before it can gain access to the inner fibres.

It is conceivable that the exchange of K^+ is to some extent determined by diffusional delays also. This would imply that even in the K^+-free incubation medium, it cannot be excluded that the concentration of K^+ between the fibres is high enough to interfere with the binding of ouabain in such a way that the apparent dissociation constant is increased. It should be added that it has not yet been determined which membrane surfaces are involved in the binding of ouabain. It was found that in frog muscle, up to 80% of the K^+ exchange may take place via the T-tubules [34], and it was recently suggested that the major part of the active Na^+ extrusion from rat extensor digitorum longus muscles takes place via the system of sarcoplasmic tubules [35]. If the walls of the T-tubules are capable of binding ouabain, the microenvironment in which the binding takes place may be even more difficult to clear for K^+.

The stimulating effect of unlabelled ouabain, digoxin, 2,4-dinitrophenol or K^+

on the wash-out of [^{3}H]ouabain may also be a consequence of the diffusional delay in the microenvironment of the ouabain-binding sites. The experiments with ($Na^+ + K^+$)-activated ATPase indicate that the process of ouabain release per se is not affected by an excess of unlabelled ouabain, and it seems likely, therefore, that the acceleration of [^{3}H]ouabain release induced by the addition of unlabelled ouabain is due to competitive inhibition of the return of labelled ouabain to its binding sites. Under basal conditions, however, it could be calculated that the concentration of [^{3}H]ouabain in the wash-out medium should not exceed 10^{-13} M during the slow phase of wash-out. Within a 10-min wash-out period, the muscle would at most be able to reaccumulate 0.5% of the [^{3}H]ouabain present in the free solution, and it seems likely, therefore, that the reattachment of labelled ouabain for a major part takes place from a microenvironment with a much higher [^{3}H]ouabain concentration.

For the calculation of the number of sites per μm^2, it was assumed that binding takes place to the outer membrane merely, and that fat cells and soleus muscle fibres can be considered as spheres and cylinders, respectively. The number of sites per μm^2 of the soleus muscle seems to be high compared with the values estimated for non-myelinated nerve fibres from rabbits [36] and for squid giant axons [14].

It would appear that in soleus muscle the number of Na^+ and K^+ transported per ouabain-binding site is relatively low. However, in muscles which had been partially depleted for K^+ by prolonged incubation in K^+-free buffer, the ouabain-suppressible rate of ^{42}K accumulation measured immediately upon readministration of K^+ was almost 3 times the control rate (0.657 ± 0.037 μmoles/g per min as compared to 0.233 ± 0.020 μmoles/g per min). Thus, although it is possible to stimulate the active Na^+–K^+ transport over and above its basal rate, it is still working far from its maximal capacity. In the isolated ($Na^+ + K^+$)-activated ATPase, the Na^+- and the K^+-sensitive sites are probably both exposed simultaneously to the ions in the incubation medium, and when Na^+ and K^+ are present at optimum concentrations (normally 130 and 20 mM, respectively [37]), the system is activated to a degree not normally encountered under basal conditions in an intact cell. Assuming that 3 Na^+ are transported per ATP molecule split and that 7000 molecules of ATP are split per ouabain-binding site per min, it can be calculated that the transport rate of 500 Na^+ per site per min measured at 30 °C correspond to 2.4% of the maximum activity of the system as measured at 37 °C.

Assuming that 2 K^+ are pumped per molecule of ATP split in the fat cells, and that 7000 molecules of ATP are split per ouabain-binding site, the accumulation of 3450 K^+ per site per min correspond to 25% of the maximum activity of the system. It should be noted that the measurements of ^{42}K influx were performed at 37 °C.

These values seem acceptable in view of the observation [38] that in ghosts of fat cells, it was possible to stimulate the rate of the ouabain-sensitive component of ^{42}K uptake by 5–6-fold by depleting the vesicles for K^+. Furthermore, in HeLa cells, a Na^+ efflux of 3600 ions per ouabain-binding site per min was found [39].

ACKNOWLEDGEMENT

The skilled technical assistance of Annemarie Krogh, Kirsten Kvist and Karin Skovgård Sørensen is gratefully acknowledged.

REFERENCES

1 Schatzman, H. J. (1953) Helv. Physiol. Pharmacol. Acta 11, 346–354
2 Glynn, I. M. (1957) J. Physiol. London 136, 148–173
3 Baker, P. F., Foster, R. F., Gilbert, P. S. and Sham, T. I. (1971) J. Physiol. London 219, 487–506
4 Touabi, M. and Jeanrenaud, B. (1970) Biochim. Biophys. Acta 202, 486–495
5 Dahl-Hansen, A. B. and Clausen, T. (1973) Biochim. Biophys. Acta 318, 147–153
6 Skou, J. C. (1960) Biochim. Biophys. Acta 42, 6–23
7 Albers, R. W., Koval, G. J. and Siegel, G. J. (1968) J. Mol. Pharmacol. 4, 324–336
8 Matsui, H. and Schwartz, A. (1968) Biochim. Biophys. Acta 151, 655–663
9 Hansen, O. (1971) Biochim. Biophys. Acta 233, 122–132
10 Ellory, C. and Keynes, R. D. (1969) Nature 221, 776
11 Hoffman, J. F. (1969) J. Gen. Physiol. 54, 343–353s
12 Baker, P. F. and Willis, J. S. (1970) Nature 226, 521–523
13 Baker, P. F. and Willis, J. S. (1972) J. Physiol. London 224, 441–462
14 Baker, P. F. and Willis, J. S. (1972) J. Physiol. London 224, 463–475
15 Caldwell, P. C. and Keynes, R. D. (1959) J. Physiol. London 148, 8P–9P
16 Perrone, J. R. and Blostein, R. (1973) Biochim. Biophys. Acta 291, 680–689
17 Kohn, P. G. and Clausen, T. (1971) Biochim. Biophys. Acta 225, 277–290
18 Cohen, P. P. (1951) in Manometric Techniques and Tissue Metabolism, (Umbreit, W. W., Burris, R. H. and Stauffer, J. F., eds), 2nd edn, p. 119, Burgess Publishing Co., Minneapolis
19 Bray, G. A. (1960) Anal. Biochem. 1, 279–285
20 Anderson, L. E. and McClure, W. O. (1973) Anal. Biochem. 51, 173–179
21 Clausen, T. (1969) Biochim. Biophys. Acta 183, 625–634
22 Rodbell, M. (1964) J. Biol. Chem. 239, 375–380
23 Gliemann, J., Østerlind, K., Vinten, J. and Gammeltoft, S. (1972) Biochim. Biophys. Acta 286, 1–9
24 Dole, V. P. and Meinertz, H. (1960) J. Biol. Chem. 235, 2575–2599
25 Siegbahn, K. (1946) Phys. Rev. 70, 127
26 Skou, J. C. and Hilberg, C. (1969) Biochim. Biophys. Acta 185, 198–219
27 Hansen, O. and Skou, J. C. (1973) Biochim. Biophys. Acta 311, 51–66
28 Akera, T. and Brody, T. M. (1971) J. Pharmacol. Exp. Ther. 176, 545–557
29 Allen, J. C., Harris, R. A. and Schwartz, A. (1971) Biochem. Biophys. Res. Commun. 42, 366–370
30 Matsui, H. and Schwartz, A. (1966) Biochim. Biophys. Acta 128, 380–390
31 Pitts, B. J. R., Lane, L. K. and Schwartz, A. (1973) Biochem. Biophys. Res. Commun. 53, 1060–1066
32 Sulakhe, P. V., Fedelesova, M., McNamara, D. B. and Dhalla, N. S. (1971) Biochem. Biophys. Res. Commun. 42, 793–800
33 Letarte, J. and Renold, A. E. (1968) in Protides of the Biological Fluids, Vol. 15, pp. 241–249 Elsevier, Amsterdam
34 Almers, W. (1972) J. Physiol. London 225, 57–83
35 Rogus, E. and Zierler, K. L. (1973) J. Physiol. London 233, 227–270
36 Landowne, D. and Ritchie, J. M. (1970) J. Physiol. London 207, 529–537
37 Skou, J. C. (1972) Bioenergetics 4, 203–232
38 Clausen, T. and Rodbell, M. (1969) J. Biol. Chem. 244, 1258–1262
39 Boardman, L. J., Lamb, J. F. and McCall, D. (1972) J. Physiol. London 225, 619–635