J. Physiol. (1975), **252**, *pp.* 43–58
With 4 text-figures
Printed in Great Britain

EFFECT OF INSULIN UPON MEMBRANE-BOUND ($NA^+ + K^+$)-ATPASE EXTRACTED FROM FROG SKELETAL MUSCLE

BY WAYNE A. GAVRYCK, RICHARD D. MOORE AND ROGER C. THOMPSON

From the Biophysics Laboratory, Department of Biological Sciences State University of New York, College of Arts and Science, Plattsburgh, New York 12901, U.S.A.

(*Received 17 December 1974*)

SUMMARY

1. Insulin stimulates the activity of membrane-bound ATPase isolated from frog skeletal muscle and from rat brain. The increase in activity of the membrane-bound ATPase system isolated from frog ranged from 9·8 to 53% at concentrations of Na^+ (25 mM), K^+ (10 mM), and ATP (2 mM) similar to those in *in vivo* experiments conducted previously (Moore, 1973). The increased activity of the membrane-bound ATPase is, therefore, at least as great as the insulin-induced increase in Na efflux (10–38%) from intact cells (Moore, 1973). If the concentration of Na^+ is lowered to 4 mM and that of ATP lowered to 0·5 mM, the increase in ouabain-inhibitable ATPase activity can reach as high as 400%.

2. Ouabain, at a concentration (10^{-3} M) sufficient to inhibit stimulation of the frog ATPase by increasing Na from 4 to 25 mM, completely blocked the stimulation of ATPase activity due to insulin.

3. At 2 mM-ATP, 100 mM-Na^+, and 20 mM-K^+, conditions which maximally activate the ($Na^+ + K^+$)-ATPase, insulin did *not* increase the ATPase, activity. Stimulation was consistently seen at 10 mM-K^+, 0·5 mM-ATP, and either 4 mM or 25 mM-Na^+.

4. The finding that insulin does not stimulate the ATPase activity in conditions in which the ($Na^+ + K^+$)-ATPase component is maximally activated and especially the fact that ouabain can reproducibly inhibit insulin stimulation of the membrane-bound ATPase activity strongly suggest that interaction of insulin with its receptor upon the plasma membrane somehow stimulates the ($Na^+ + K^+$)-ATPase system (ouabain sensitive; ATP phosphohydrolase, EC (3.6.1.3). These results are consistent with previous studies of the effect of insulin upon Na efflux from intact cells (Moore, 1973) and support the previous conclusion that the

component of Na efflux stimulated by insulin is active. The evidence suggests that insulin probably does not affect V_{max} of the $(Na^+ + K^+)$-ATPase system, but may increase the affinity of the enzyme system to one or more effectors, most likely Na^+, ATP, and perhaps K^+.

5. Oxidized glutathione ($2{\cdot}7 \times 10^{-6}$ M), 10^{-6} M albumin, and 10^{-6} M, 10^{-7} M, and 10^{-8} M cyclic AMP did not affect the ATPase activity.

6. The results are consistent with the view that the Na pump, $(Na^+ + K^+)$-ATPase, is intimately involved with the physiological action of insulin and may be a transducer between the binding of insulin to its receptor on the plasma membrane and the cellular actions of insulin.

INTRODUCTION

Insulin stimulates Na efflux from frog skeletal muscle (Moore, 1973). The following facts suggested that the increment in Na efflux produced by insulin was active, that is, was due to a stimulation of the Na pump. Firstly insulin stimulated net Na efflux as well as one-way ^{22}Na efflux; secondly both of these effects were blocked by 5×10^{-6} M acetylstrophanthidin; finally K-free Ringer inhibited the effect of insulin upon ^{22}Na efflux (Moore, 1973).

Zierler, Rogus & Hazelwood (1966) have shown that insulin decreases intracellular Na in muscles from hypophysectomized rats, and Creese (1968) has reported that insulin increased ^{22}Na efflux from isolated rat hemidiaphragms. However, on the basis of kinetic studies of Na efflux from muscle not treated with insulin, Zierler (1972) has argued that the effect of insulin upon Na efflux is not upon an active efflux from the cytoplasm, but upon Na movement from some other compartment, presumably the sarcoplasmic reticulum, with the insulin effect being entirely upon passive fluxes. Zierler bases this argument partly upon the report (Rogus, Price & Zierler, 1969) that insulin did not stimulate $(Na^+ + K^+)$-ATPase (ouabain sensitive; ATP phosphohydrolase, EC 3.6.1.3) isolated from rat skeletal muscle. Letarte & Renold (1969) stated that insulin did not affect the activity of $(Na^+ + K^+)$-ATPase isolated from fat cells, although none of the experimental conditions were specified.

More recently, other laboratories have demonstrated an effect of insulin upon membrane-bound ATPase, although the effect of inhibitors considered specific for $(Na^+ + K^+)$-ATPase, such as ouabain, were not used to help establish the identity of the ATPase. For example, Hadden, Hadden, Wilson & Good (1972) have reported that insulin stimulates an ATPase activity in membrane fragments from human lymphocytes. This stimulation occurred in the presence of 5 mM-Mg^{2+}, 5 mM-Ca^{2+}, or 5 mM-Mg^{2+} plus 100 mM-Na^+ and 20 mM-K^+. Jarett & Smith (1974) have

reported that insulin stimulates a membrane-bound ATPase isolated from rat adipocytes.

The experiments reported here demonstrate that insulin stimulates a membrane-bound ATPase isolated from frog skeletal muscle and from rat brain. In both preparations, the effect of insulin can be completely inhibited by ouabain, suggesting that the identity of this ATPase is the $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) generally accepted as being associated with, if not identical to, the sodium pump. Oxidized glutathione, albumin, and cyclic AMP do not have an effect upon the frog ATPase activity.

METHODS

Most of these experiments were conducted using membrane-bound ATPase extracted from frog (*Rana pipiens*) skeletal muscle. The animals were decapitated and their hind legs removed. In 1970, Boegman, Manery & Pinteric reported a method of isolation from frog skeletal muscle of membrane-bound ATPase preparation which was synergistically activated by $Na^+ + K^+$ and was inhibited by ouabain. We have used a modification of this method to extract membrane-bound ATPase from frog skeletal muscle with the essential modifications consisting of elimination of the final sucrose gradient centrifugation, suspension of the final pellet in 1·0 mM-H_4EDTA neutralized with Tris, and storage for 6–9 days at $-6°$ C. The specific activity of the enzyme preparation (μM P_i/mg protein.hr) ranged from 5 to 12 μ. with the % activity due to $Na^+ + K^+$ activation averaging 75% (ranging from 56 to 92%) of the total ATPase activity. For comparison of species and tissue effect, some experiments were conducted using a membrane-bound ATPase kindly supplied by Dr Amir Askari and extracted from rat brain by the method of Skou (1962). The specific activity of the rat brain preparation was 80 μ.; 90% of the activity required 100 mM-Na^+ + 20 mM-K^+ and was inhibited by ouabain, the other 10% being Mg-ATPase.

All hydrolysis solutions contained 5 mM-$MgCl_2$ regardless of the concentration of ATP which was either 2, 0·5 or 0·1 mM as indicated. Na^+ or K^+ was present in the concentrations indicated under Results. Tris was used as a buffer and also as a substitute for Na^+ and K^+, with the total concentration of cation always being 196 mM. Insulin was always dissolved in the hydrolysis solution for 1–1·5 hr before the experiment. The calculated concentration of insulin was 400 m-u./ml. in each experiment. At 36° C, the pH was 7·1.

All tubes were placed in a 36° C water-bath for 10–15 min before the initiation of the reaction by the addition of 0·1 ml. of the enzyme suspension, the enzyme having been thawed at 22° C immediately before use. The hydrolysis was always carried out at 36° C in a total volume of 2 ml. and the reaction was stopped by precipitating the enzyme with 1·0 ml. cold (0° C) 15% TCA and immediately placing the tubes on ice. In all cases, three tubes of hydrolysis solution containing ATP and all other components except the enzyme were run through the same procedure, including incubation at 36° C, and used as blanks to determine contaminating P_i and any P_i produced by non-enzymatic hydrolysis. This P_i was subtracted from the net amounts to determine the amount of P_i due to enzymatic activity. The extent of hydrolysis of the ATP was measured by determining P_i by the isobutanol-benzene extraction method of Swanson, Bradford & McIlwain (1964) in the preliminary experiments, with subsequent determinations (after Table 1) being conducted by the method of Fiske & Subbarow (1925). In all experiments

conducted with 2 mM-ATP, the total P_i released did not exceed 6% of the terminal phosphate of ATP. At 0·5 mM-ATP, the maximum P_i released was usually 8–10% with a maximum of 40%, and at 0·1 mM, the maximum released was 32% of the terminal phosphate of ATP. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). At ATP concentrations of 2 and 0·5 mM, the hydrolysis was linear for 1 hr; at 0·1 mM-ATP, the reaction began to deviate from linearity at 30–40 min. Accordingly, all reactions at 2 mM-ATP were run for 45 min and those at 0·5 or 0·1 mM were run for 30 min.

All chemicals were reagent grade. All water was deionized two times in nalgene containers. In all experiments, Na_2ATP was used and in all cases this was obtained from Schwartz Biochemicals. Except for the experiments in Table 1, this was the ultrapure grade. Albumin, fraction V; oxidized glutathione, and cyclic AMP were obtained from Sigma. The insulin was low Zn (0·03% Zn) beef amorphous insulin in the preliminary experiments but because of discontinuance of the supply, all other experiments were conducted using Porcine insulin (0·00% Zn), 25·9 u./mg. The insulin and ouabain were a gift from Eli Lilly.

RESULTS

Effect of insulin upon membrane-bound ATPase

In five of seven preliminary experiments conducted at 25 mM-Na^+, 10 mM-K^+, and 2 mM-ATP, beef insulin produced a statistically significant ($P < 0·05$) increase in total frog skeletal muscle membrane ATPase activity ranging from 9·8 to 53% (mean 24·6%). This concentration of Na^+ and of K^+ was chosen because it approximated the initial intracellular Na^+ concentration (26 mM) and the extracellular concentration of K^+ (10 mM) used in experiments demonstrating an effect of insulin upon active Na efflux from intact frog sartorius muscle (Moore, 1973). In the remainder of the experiments, we found that insulin produced a statistically significant increase in ATPase activity in over 90% of the enzyme preparations from frog skeletal muscle.

In order to rule out the possibility that the stimulation of the ATPase activity was due to a non-specific peptide effect and the possibility that the effect might simply be due to the presence of a disulfide linkage in a peptide structure, oxidized glutathione was tested. $2·7 \times 10^{-6}$ M oxidized glutathione failed to produce an effect upon a frog muscle ATPase preparation which did respond to insulin (+18%, $P < 0·05$) at the same concentration. In addition, 10^{-6}, 10^{-7}, and 10^{-8} M cyclic AMP did not produce a significant effect upon the enzyme. In another experiment, we also found that 10^{-6} M albumin produced no effect upon frog membrane-bound ATPase activity at 25 mM-Na^+ and 10 mM-K^+.

Effect of ouabain

Fig. 1 demonstrates that 10^{-4} M ouabain failed to produce a clear inhibition of the stimulation of ATPase activity by insulin at 25 mM-Na^+, 10 mM-K^+, and 2 mM-ATP. This same type of experiment was also conducted at an ATP concentration of 0·5 mM. In this latter case, illustrated in Fig. 2, ouabain *did* produce a statistically significant decrease in the stimulation of the ATPase activity by insulin at 25 mM-Na^+ and

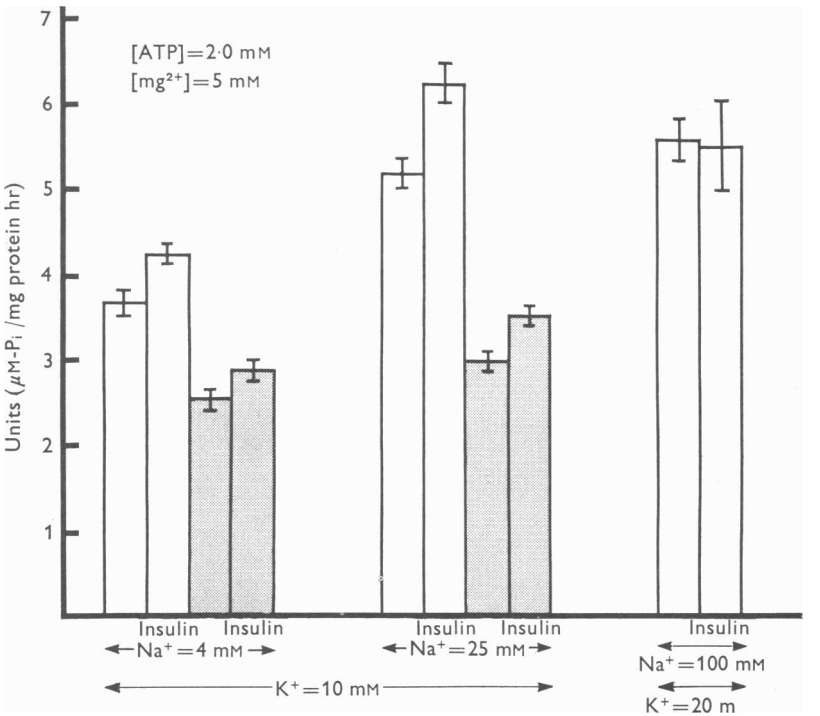

Fig. 1. The effect of 10^{-4} M ouabain and of Na^+ at 2 mM-ATP upon the response of the membrane-bound ATPase isolated from frog skeletal muscle. The shaded columns indicates the presence of 10^{-4} M ouabain. Based upon total ATPase activity, insulin produced a 15% ($P < 0{\cdot}005$) increase at 4 mM-Na^+, 10 mM-K^+; a 20% ($P < 0{\cdot}005$) increase at 25 mM-Na^+, 10 mM-K^+, and no change (+0·9%, $P > 0{\cdot}5$) at 100 mM-Na^+, 20 mM-K^+, and 2 mM-ATP. In the presence of 10^{-4} M ouabain, insulin still produced an increment in total ATPase activity at 4 mM-Na^+, 10 mM-K^+ of 14% ($P < 0{\cdot}05$) and at 25 mM-Na^+, 10 mM-K^+, of 20% ($P < 0{\cdot}005$). In the presence of ouabain, elevation of Na^+ from 4 to 25 mM produced a 17% increase ($P < 0{\cdot}005$) based upon total ATPase activity. The specific activity of the enzyme is plotted in units which are defined as μM-P_i produced per hr per mg protein of the membrane preparation. Each column is the average ± S.D. of seven determinations.

10 mM-K^+; this inhibition was almost (95%) complete. However, it is of some interest that in the same experiment, ouabain at 4 mM-Na^+ and 10 mM-K^+ produced only about a 50% reduction, which was not quite significant ($P > 0.05$), in the response to insulin. It is possible that the reason ouabain in this experiment was effective at 25 mM-Na^+, and only questionably so at 4 mM-Na^+, is the fact that the ability of ouabain to inhibit the ($Na^+ + K^+$)-ATPase enzyme system in the presence of Mg^{2+} and ATP is strongly dependent upon the concentration of Na^+ (Skou, Butler & Hansen, 1971), most likely because in the presence of both Mg^{2+}

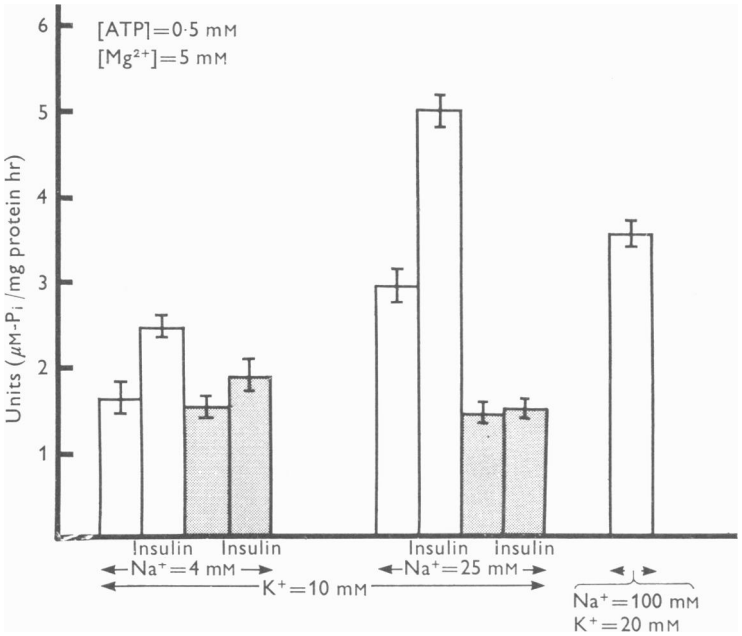

Fig. 2. The effect of 10^{-4} M ouabain and of Na^+ at 0·5 mM-ATP upon the response of the membrane-bound ATPase isolated from frog skeletal muscle. The shaded columns indicate the presence of 10^{-4} M ouabain. Based upon total ATPase activity, insulin produced a 50% ($P < 0.005$) increase at 4 mM-Na^+, 10 mM-K^+, and a 70% ($P < 0.005$) stimulation at 25 mM-Na^+, 10 mM-K^+. In the presence of 10^{-4} M ouabain, and in the presence of 4 mM-Na^+, the stimulation due to insulin was decreased to 24%, but was still significant ($P < 0.025$). However, at 25 mM-Na^+, the effect of insulin was effectively (95%) inhibited, being reduced to a 3% stimulation which was not significant ($P > 0.5$). Assuming that insulin stimulated only the ($Na^+ + K^+$)-ATPase component, the stimulation at 4 mM-Na^+ was 429% and at 25 mM-Na^+ was 147%. In the presence of ouabain, elevation of Na^+ from 4 to 25 mM produced no effect (+3·6%, $P > 0.5$) upon ATPase activity. The effect of maximal cation ($Na^+ + K^+$) activation is illustrated for comparison. Each column represents the mean ± S.D. of seven determinations.

and ATP, the binding of ouabain to the enzyme system is strongly dependent upon Na^+ (Lane, Copenhaver, Lindenmayer & Schwartz (1973).

Comparison of Fig. 1 with Fig. 2 provides a possible explanation for the finding that ouabain inhibited the stimulation by insulin in one experiment, but not in the other. In Fig. 1, not only did 10^{-4} M ouabain fail to inhibit the stimulation of the enzyme by insulin, but it also failed to completely inhibit the stimulation of the enzyme produced by increasing the concentration of Na^+ from 4 to 25 mM. This suggestion is reinforced by the results illustrated in Fig. 3 where the same experimental format was conducted at 0·1 mM-ATP. Again, 10^{-4} M ouabain failed to inhibit the stimulation of the enzyme by insulin when the concentration of ouabain (10^{-4} M) was not sufficient to prevent a stimulation by increasing Na^+ from 4 to 25 mM.

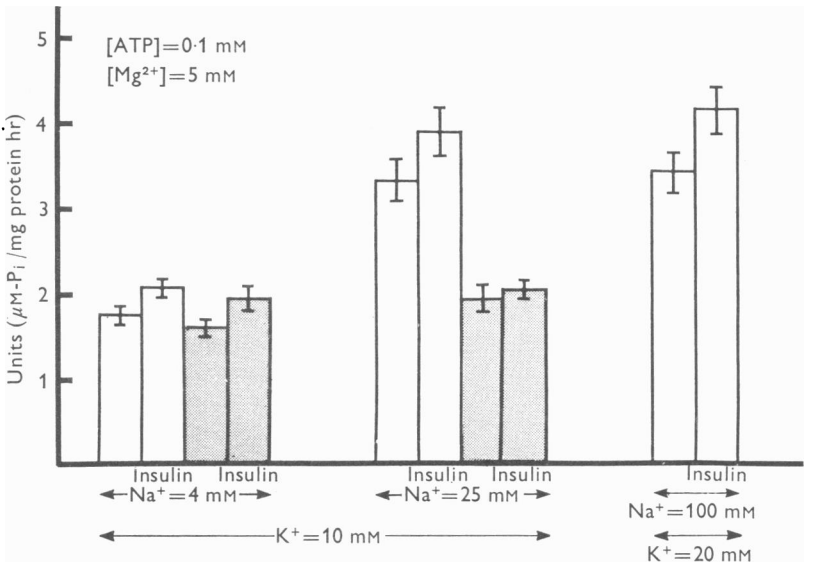

Fig. 3. The effect of 10^{-4} M ouabain and of Na^+ at 0·1 mM-ATP upon the response of the membrane-bound ATPase isolated from frog skeletal muscle. The shaded columns indicate the presence of 10^{-4} M ouabain. Based upon total ATPase activity, insulin produced a 17% ($P < 0.005$) increase at 4 mM-Na^+, 10 mM-K^+, and a 17% increase ($P < 0.05$) at 25 mM-Na^+, 10 mM-K^+. At 100 mM-Na^+, 20 mM-K^+, and 0·1 mM-ATP, insulin produced a 22% increase ($P < 0.025$) in total ATPase activity. 10^{-4} M ouabain had little effect at 4 mM-Na^+, 10 mM-K^+ with insulin still producing a 20% ($P < 0.025$) stimulation. At 25 mM-Na^+, 10 mM-K^+, 10^{-4} M ouabain reduced the increment in ATPase activity to a not quite significant 9·4% ($P < 0.10$). In the presence of ouabain, elevation of Na^+ from 4 to 25 mM produced a 19% increase in total ATPase activity ($P < 0.005$). Each column represents the mean ± S.D. of seven determinations.

The above results, plus previous reports that the $(Na^+ + K^+)$-ATPase system isolated from toad bladder is not completely inhibited by ouabain at concentrations as high as 10^{-4} M (Bonting & Canady, 1964; Cortas & Walser, 1971) suggested using a higher concentration of ouabain (10^{-3} M) to inhibit more completely the $(Na^+ + K^+)$-ATPase. Fig. 4 illustrates one of two experiments testing the effect of 10^{-3} M ouabain: in both experiments, insulin produced a statistically significant stimulation of ATPase activity which at 25 mM-Na^+ and 10 mM-K^+ was significantly inhibited by 10^{-3} M ouabain. In Fig. 4, the ouabain inhibition of the insulin effect was essentially complete, with the increment in total ATPase activity due to insulin being reduced 93% by 10^{-3} M ouabain. It is of considerable interest that in both of these experiments, not only was the stimulation due to insulin inhibited by 10^{-3} M ouabain, but the stimulation due to raising Na^+ from 4 to 25 mM was also very much reduced and was no longer statistically significant, supporting the thesis proposed above to account for those cases where 10^{-4} M ouabain failed to block the stimulation by insulin.

Although the inhibition of the insulin effect in Fig. 4 was essentially complete at 25 mM-Na^+ and 10 mM-K^+, the prevention of the insulin effect was not quite complete at 100 mM-Na^+ and 20 mM-K^+. This may be due to the fact that elevation of K^+ tends to decrease ouabain binding (Lindenmayer & Schwartz, 1973).

For basis of comparison, two similar experiments were conducted using the membrane-bound ATPase extracted from rat brain. Not only did insulin produce a statistically significant increase (25%, $P < 0.05$; 21%, $P < 0.005$) in ATPase activity at 25 mM-Na^+, 10 mM-K^+ and 0·5 mM-ATP, but under these conditions 10^{-3} M ouabain produced a complete inhibition of the insulin effect. At 10^{-4} M, ouabain did not prevent a statistically significant stimulation by insulin, although the absolute magnitude of the increment of ATPase activity due to insulin was reduced by 60%.

Effect at 100 mM-Na^+ and 20 mM-K^+

In five out of six experiments conducted at 2 mM-ATP, 100 mM-Na^+ and 20 mM-K^+ (conditions which approach maximal activation of this ATPase preparation), insulin did not produce a statistically significant increase in ATPase activity, even though the same enzyme preparations had been responsive to insulin at lower concentrations of ATP, Na^+, and/or K^+. In the remaining experiment, the insulin effect was small (+9%, $P < 0.05$). When $[K^+]$ was lowered to 10 mM, insulin produced a statistically significant (13%, $P < 0.05$) increase in frog ATPase activity in the presence of 100 mM-Na^+ and 2 mM-ATP.

Effect of ATP

Inspection of Figs. 3 and 4 demonstrates that at 0·1 and at 0·5 mM-ATP, insulin still produced a statistically significant stimulation of frog membrane-bound ATPase activity in the presence of amounts of Na^+

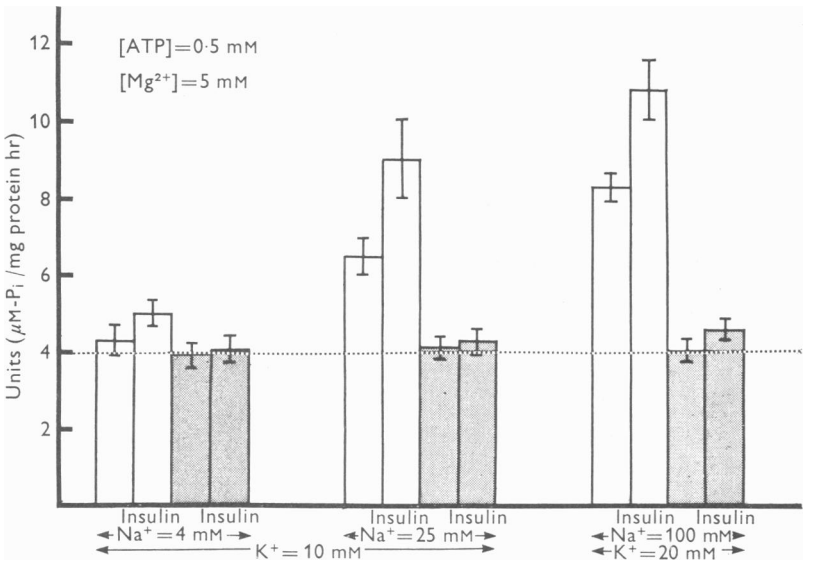

Fig. 4. The effect of 10^{-3} M ouabain and of Na^+ at 0·5 mM-ATP upon the response of membrane-bound ATPase isolated from frog skeletal muscle. Shaded columns indicate the presence of 10^{-3} M ouabain. Based upon total ATPase activity, insulin produced a 14% increase ($P < 0{\cdot}005$) at 4 mM-Na^+, 10 mM-K^+ and a 36% stimulation ($P < 0{\cdot}005$) at 25 mM-Na^+, 10 mM-K^+. The presence of 10^{-3} M ouabain reduced the effect of insulin at 25 mM-Na^+ ,10 mM-K^+ to +5% ($P > 0{\cdot}25$), a 93% reduction of the increment due to insulin, indicating a statistically significant inhibition of the insulin effect. In the presence of 10^{-3} M ouabain, elevation of Na^+ from 4 to 25 mM did not produce a significant effect (+6%, $P > 0{\cdot}5$). At 100 mM-Na^+, 20 mM-K^+, and 0·5 mM-ATP, insulin produced a 30% ($P < 0{\cdot}005$) increase in total ATPase activity. Under these conditions, 10^{-3} M ouabain resulted in an 85% decrease in the increment due to insulin although the stimulation by insulin (+15%, $P < 0{\cdot}05$) was just statistically significant. The dotted line represents Mg-ATPase (ouabain-uninhibitable ATPase) activity; activity above that line is presumed to be due to $(Na^+ + K^+)$-ATPase. Each column represents the mean ± S.D. of six determinations.

and K^+ (100 mM-Na^+ and 20 mM-K^+) sufficient to approach maximal cation activation of the enzyme. The observation that this occurs at 0·1 mM-ATP was confirmed in another experiment, as was the observation at 0·5 mM-ATP.

The fact that at 100 mM-Na^+ and 20 mM-K^+, insulin stimulated the ATPase activity only if [ATP] was lowered suggests that the response of the enzyme system to insulin is dependent upon [ATP]. Indeed, inspection of Figs. 1–4 will reveal that the effect of insulin was more pronounced as [ATP] was lowered. Another experiment designed to establish this

dependency upon [ATP] was conducted at 4 mM-Na^+ and 20 mM-K^+. This experiment demonstrated that when [ATP] was dropped from 0·5 to 0·1 mM, the stimulation due to insulin increased from 10 % ($P < 0·05$) to 70 % ($P < 0·025$) of the ouabain-inhibitable fraction of the ATPase activity, with even the absolute magnitude of insulin stimulation increasing from 0·15 to 0·21 u. ($P < 0·05$). When this experiment was repeated at 25 mM-Na^+ and 10 mM-K^+, a decrease in [ATP] from 4 to 2 mM produced no change in the response to insulin (+57 % *vs.* 60 %, $P < 0·005$), but a further decrease to 0·5 mM-ATP resulted not only in a percentage increase in the response of the ouabain-inhibitable fraction to insulin (+217 %, $P < 0·005$), but in an almost doubling (83 % increase) of the absolute magnitude of insulin stimulation.

DISCUSSION

Identity of the membrane-bound ATPase

Present studies. Several aspects of the above results suggest that the ATPase activity stimulated by insulin is that traditionally considered to be associated with, if not identical to, the Na pump, i.e. the ($Na^+ + K^+$)-ATPase (EC 3.6.1.3). The concentration of insulin which stimulated the activity of membrane-bound ATPase isolated from frog skeletal muscle was within the same range as that which had previously been shown to stimulate Na efflux from intact frog skeletal muscle (Moore, 1973). Ouabain completely blocked the stimulation of the ATPase activity provided the concentration of ouabain was sufficient (10^{-3} M) to block completely activation of the enzyme system by Na^+. The stimulation of the ATPase by insulin was eliminated or at least very much diminished when the concentrations of effectors for the ($Na^+ + K^+$)-ATPase (K^+, and especially Na^+ and ATP) were sufficiently high to produce maximal activation of the enzyme system. Finally the magnitude of the increase in total ATPase activity due to insulin (9·8–53 % at 2 mM-ATP) is at least as great as the increment of Na efflux (10–38 %) produced by insulin in intact cells from the same tissue, frog skeletal muscle. If one considers that the increment due to insulin is most likely limited to the ($Na^+ + K^+$)-ATPase component (i.e. if one subtracts the Mg^{2+}-ATPase, or ouabain uninhibitable component) the stimulation is much greater than 9·8–53 %. This means that the stimulation produced by insulin of the energy transduction mechanism (as reflected by the hydrolysis of ATP by the ($Na^+ + K^+$)-ATPase system) is more than sufficient to account for the increment in Na efflux and supports the hypothesis that stimulation of Na efflux from frog skeletal muscle can be entirely accounted for by an effect upon the sodium pump, i.e. the ($Na^+ + K^+$)-ATPase system.

Other studies. In view of the present studies, the conclusion that the adipocyte plasma membrane ATPase stimulated by insulin is Mg^{2+}-ATPase, and not ($Na^+ + K^+$)-ATPase (Jarett & Smith, 1974), may require reexamination. This conclusion was based upon the fact that at high concentrations of effectors (138 mM-Na^+, 5 mM-K^+, and 3·5 mM-ATP), insulin did not increase the ATPase activity; ouabain was not used. The present results suggest that insulin would not be expected to stimulate the ($Na^+ + K^+$)-ATPase system under these high concentrations. Rogus *et al.* (1969) also tested the effect of insulin at concentrations of Na^+, K^+, and ATP which maximally activated the ($Na^+ + K^+$)-ATPase they prepared from rat skeletal muscle. This same comment applies to the work of Hadden *et al.* (1972) who found a smaller stimulation by insulin of human lymphocyte plasma membrane ATPase activity in 2 mM-ATP at 100 mM-Na^+, 20 mM-K^+, and 5 mM-Mg^{2+} than at either 5 mM-Mg^{2+} or Ca^{2+} alone.

On the other hand, it might seem that the finding that insulin stimulated membrane-bound ATPase in the presence of Mg^{2+} (Jarett & Smith, 1974; Hadden *et al.* (1972) or of Ca^{2+} (Hadden *et al.* 1972), but without added Na^+ and K^+, would indicate that the ATPase in these two studies was not ($Na^+ + K^+$)-ATPase. This conclusion does not necessarily follow. For example, a small amount of bound Na^+ might be present; also the possibility should be considered (Moore, 1973) that insulin increases the affinity of the ($Na^+ + K^+$)-ATPase system toward not only Na^+, but toward H^+. This would be consistent with the report that H^+ may be able to substitute for Na^+ in activating the ($Na^+ + K^+$)-ATPase system (Fujita, Nagano, Mizuno, Tashima, Nakao & Nakao, 1968). Moreover, Goldfarb & Rodnight (1970) have presented evidence suggesting that even by repeated washings, it is nearly impossible to remove bound K^+ from membrane fragments sufficiently to inactivate all of the ($Na^+ + K^+$)-ATPase activity.

In view of these possible complexities, it seems essential to use ouabain, as demonstrated in the present experiments, to identify the nature of the ATPase which is stimulated by insulin.

Physiological significance

Insulin concentration and physiological effects. The concentration of insulin (400 m-u./ml.) used in the present experiments is considerably higher than mammalian blood levels. However, where albumin is not used to prevent adsorption to glassware, it is the same concentration which produces such physiological responses in frog skeletal muscle as increased uptake of 3-O-methyl-glucose (Narahara & Ozand, 1963) and also increased production of lactate (Ozand & Narahara, 1964).

This concentration was chosen because it is near the value which gives maximal stimulation of active Na efflux from frog skeletal muscle (Moore, 1973). In both studies, the comparatively high concentration of insulin required may have been

due to species differences and/or to adsorption to glassware. So as to avoid possible artifacts in these initial experiments, neither gelatin nor albumin was used to prevent adsorption of insulin to glassware.

There are other findings which suggest that it is likely that the effects of insulin described here and upon active Na efflux (Moore, 1973) have a role in the physiological action of insulin. As early as 1923 (Briggs, Kiechig, Doisy & Weber) it was known that insulin decreases serum K^+ concentration. By infusion of insulin into the brachial artery of normal human males, Zierler & Rabinowitz (1964) have shown that this decrease in serum K^+ is due to an uptake of K^+ by adipose tissue and especially by muscle. Elevation of plasma insulin by as little as 38 μu./ml. was sufficient to stimulate K^+ uptake while producing no measurable effect upon glucose uptake.

The present results as well as the demonstration that insulin stimulates active Na efflux (Moore, 1973), and the absence of any known mechanism other than the Na pump to produce K^+ uptake, support the thesis that the effect of micromolar concentrations of insulin upon K^+ uptake in mammals is due to a stimulation of the Na pump.

The plausibility that physiological concentrations of insulin stimulate the Na pump *in vivo* is strengthened by the finding (Nizet, Lefebvre & Crabbé, 1971) that 350 μu./ml. produced a very significant increase in tubular reabsorption of Na^+ in the isolated dog kidney and by the finding that insulin therapy is associated with Na retention (Saudek, Boulter, Knopp & Arky, 1974) in the human being.

The fact that insulin also stimulated ($Na^+ + K^+$)-ATPase activity isolated from rat brain is of some interest in view of the general opinion that, in contrast to muscle, the brain is probably not a target organ for insulin. However, Ellison (1958) has reported that insulin affects K^+ transport in the brain.

Relevance to studies on Na transport. These results provide strong evidence against the thesis advanced by Zierler (1972) that the effect of insulin upon Na efflux is entirely secondary to an effect upon passive fluxes. Moreover, in view of the fact that insulin did not stimulate membrane-bound ATPase activity in the presence of optimal concentrations of Na^+, K^+ and ATP, it seems unlikely that the entire effect of insulin upon Na efflux is due to an 'unmasking' of pump sites as suggested by Grinstein & Erlij (1974). In addition, since insulin stimulates the ($Na^+ + K^+$)-ATPase system *in vitro*, it becomes more difficult to maintain that the entire effect of insulin upon Na efflux is secondary to an induced synthesis of new pump sites, a conclusion supported by the lack of insulin effect upon Na:Na exchange diffusion (Moore, 1973).

The present experiments also demonstrate that it is not necessary to assume that the stimulation of Na efflux by insulin is secondary to an increase in intra-

cellular ATP. Indeed, under the present experimental conditions, the insulin effect was manifest in the face of an ATP concentration which was decreasing slightly. Moreover, insulin evidently produces no change in intracellular concentrations of ATP (Walaas, Walaas & Wick, 1969) in rat diaphragm or in fat cells (Bihler & Jeanrenaud, 1970) although it does stimulate the production of ATP by glycolysis (Ozand & Narahara, 1964).

In view of the fact that 5×10^{-6} M acetylstrophanthidin produced a nearly complete inhibition of the insulin stimulation of ^{22}Na efflux, it might seem surprising that 10^{-4} M ouabain did not produce a complete inhibition of the insulin effect upon the isolated $(Na^+ + K^+)$-ATPase system from the same species and tissue. This might be explained by the fact that ouabain binds to the enzyme system relatively slowly (Lindenmayer & Schwartz, 1973). The rate of binding of acetylstrophanthidin to the $(Na^+ + K^+)$-ATPase system has not been studied. The fact that Na^+ increases the rate of ouabain binding (Lindenmayer & Schwartz, 1973) may explain the difference in ouabain inhibition of the insulin effect at 25 mM-Na^+ versus 4 mM-Na^+ as illustrated in Figs. 3 and 4.

Molecular significance

The role of ATP. Of considerable interest is the fact that decreasing ATP from 2 to 0·5 mM, a decrease of only fourfold, results in a substantial increase in the responsiveness of the enzyme system to insulin. As pointed out in Results, this is especially easy to see at concentrations of Na^+ and K^+ which maximally activate the enzyme system. Moreover, a comparison of experiments conducted at 2 mM (Fig. 2) and at 0·5 mM (Fig. 3) shows that the same pattern held true at 10 mM-K^+ and 4 mM-Na^+ and at 10 mM-K^+ and 25 mM-Na^+. It is doubtful that this effect involved a change in K_m of the active site for ATP hydrolysis, since it seems unlikely that the K_m for this site (Site II) on the $(Na^+ + K^+)$-ATPase system isolated from frog skeletal muscle would be much different from that reported for the enzyme system in guinea-pig kidney membranes, where the K_m for the substrate site has been reported to be 2×10^{-4} mM (Post, Hegyvary & Kume, 1972). Even the lower concentration of 0·5 mM-ATP is over three orders of magnitude greater than this value.

Supporting this interpretation is the fact that in our experiments with the enzyme system isolated from rat brain, we again observed an increase in responsiveness to insulin when ATP was lowered from 2 to 0·5 mM. K. D. Straub (personal communication) has also observed the same phenomenon when studying the effect of insulin upon the ATPase system isolated from guinea-pig brain or guinea-pig kidney, the latter being especially to the point in view of the report by Post *et al.* (1972).

Although the K_m for the active site is too low, evidence exists for a second site, an allosteric activating site (Site I), with a K_m for ATP of $\sim$ 0·1 mM (Post *et al.* 1972). Lowering the [ATP] from 2 to 0·5 mM would be expected to produce at least a partial unloading of this site. This raises the possibility that the increased stimulation of the enzyme system

by insulin at the lower [ATP] may be due to an insulin-induced change in conformation (decrease in K_m) of this site with a resulting increase in the activity of the enzyme system.

Regardless of the exact mechanism, the evidence strongly indicates that if intracellular ATP is lowered below normal levels, the response of the $(Na^+ + K^+)$-ATPase system to insulin is amplified. Since one action of insulin is to stimulate production of ATP by glycolysis (Ozand & Narahara, 1964), this suggests the possibility of a strictly molecular feedback mechanism operating to control the degree of amplification of response to a hormone.

Relevance to mechanism of insulin action. The evidence presented here supports the earlier suggestion that insulin stimulates the Na pump either by a 'direct' action, or by mechanisms within the membrane phase. This by no means implies that cyclic nucleotides are not involved in the action of insulin. However, it does support the suggestion that the Na pump is involved as a transducer in the primary effect of insulin. Not only does insulin elevate intracellular $[K^+]$ and lower intra-cellular $[Na^+]$ (Moore, 1973), but by hyperpolarizing the plasma membrane (Zierler, 1959), which is most likely secondary to its stimulation of the sodium pump, insulin shifts the equilibrium distribution of all ionic species across the plasma membrane. Such an effect would, of course, be most pronounced upon divalent ions, such as Ca^{2+} and Mg^{2+}. Indeed, Lostroh & Krahl (1973) have reported that in rat uterus insulin not only elevated intracellular K^+ but also intracellular Mg^{2+}, and had no effect upon the distribution of these ions in the presence of ouabain. The possibility of an effect upon pH_i presents an obvious means whereby insulin might affect intracellular events (Moore, 1973). For example, stimulation of glycolysis might be partially explained by an increase in intracellular pH resulting in activation of phosphofructokinase, a key enzyme in glycolysis extremely sensitive to pH elevations between 6·0 and 7·0 (Trivedi & Danforth, 1966).

Any linkage between the effect of insulin upon the sodium pump and other actions of insulin is undoubtedly complex. Nevertheless, some mechanism must exist for this coupling, since the finding that glycogen synthetase cannot be stimulated by insulin in cells treated with ouabain (Blatt, McVerry & Kim, 1972) has been confirmed by Horn, Walaas & Walaas (1973).

BIHLER, I. & JEANRENAUD, B. (1970). ATP content of isolated fat cells; effects of insulin, ouabain, and lipolytic agents. *Biochim. biophys. Acta* **202**, 496–506.

BLATT, L. M., MCVERRY, P. H. & KIM, KI-HAN (1972). Regulation of hepatic glycogen synthetase of *Rana catesbeiana*; inhibition of the action of insulin by ouabain. *J. biol. Chem.* **243**, 1957–1965.

BOEGMAN, R. J., MANERY, J. F. & PINTERIC, L. (1970). The separation and partial purification of membrane bound ($Na^+ + K^+$)-independent Mg^{2+}-ATPase from frog skeletal muscle. *Biochim. biophys. Acta* **203**, 506–530.

BONTING, S. L. & CANADY, M. R. (1964). Na-K activated adenosine triphosphatase and sodium transport in toad bladder. *Am. J. Physiol.* **207**, 1005.

BRIGGS, A. P., KIECHIG, I., DOISY, E. A. & WEBER, C. J. (1923). Some changes in the composition of blood due to the injection of insulin. *J. biol. Chem.* **58**, 721–730.

CORTAS, N. & WALSER, M. (1971). ($Na^+ + K^+$)-activated ATPase in isolated mucosal cells of toad bladder. *Biochim. biophys. Acta* **249**, 181–187.

CREESE, R. (1968). Sodium fluxes in diaphragm muscle and the effects of insulin and serum proteins. *J. Physiol.* **197**, 255–278.

ELLISON, R. J. (1958). Changes in cerebral potassium during insulin hypoglycemia. *Proc. Soc. exp. Biol. Med.* **98**, 128–129.

FISKE, C. H. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorous. *J. biol. Chem.* **66**, 375–400.

FUJITA, M., NAGANO, K., MIZUNO, N., TASHIMA, Y., NAKAO, T. & NAKAO, M. (1968). Comparison of some minor activities accompanying a preparation of sodium-plus-potassium ion-stimulated adenosine triphosphatase from pig brain. *Biochem. J.* **106**, 113–121.

GOLDFARB, P. S. G. & RODNIGHT, R. (1970). The role of bound potassium ions in the hydrolysis of low concentrations of adenosine triphosphate by preparations of membrane fragments from ox brain cerebral cortex. *Biochem. J.* **120**, 15–24.

GRINSTEIN, S. & ERLIJ, D. (1974). Insulin unmasks latent sodium pump sites in frog muscle. *Nature, Lond.* **251**, 57–58.

HADDEN, J. W., HADDEN, E. M., WILSON, E. E. & GOOD, R. A. (1972). Direct action of insulin on plasma membrane ATPase activity in human lymphocytes. *Nature, New Biol.* **235**, 174–177.

HORN, R. S., WALAAS, O. & WALAAS, E. (1973). The influence of sodium, potassium and lithium on the response of glycogen synthetase I to insulin and epinephrine in the isolated rat diaphragm. *Biochim. biophys. Acta* **313**, 296–309.

JARETT, L. & SMITH, R. M. (1974). The stimulation of adiphocyte plasma membrane magnesium ion-stimulated adenosine triphosphatase by insulin and concanavalin A. *J. biol. Chem.* **249**, 5195–5199.

LANE, L. K., COPENHAVER, J. H. Jr., LINDENMAYER, G. E. & SCHWARTZ, A. (1973). Purification and characterization of and ^{3}H-ouabain binding to the transport adenosine triphosphatase from outer medulla of canine kidney. *J. biol. Chem.* **248**, 7197–7220.

LETARTE, J. & RENOLD, A. E. (1969). Ionic effects on glucose transport and metabolism by isolated mouse fat cells incubated with or without insulin. III. Effects of replacement of Na^+. *Biochim. biophys. Acta* **183**, 366–374.

LINDENMAYER, G. E. & SCHWARTZ, A. (1973). Evidence that sodium–potassium competition modulates the rate of ouabain interactions with ($Na^+ + K^+$)-adenosine triphosphatase during enzyme catalysis. *J. biol. Chem.* **248**, 1291–1300.

LOSTROH, A. J. & KRAHL, M. E. (1973). Accumulation *in vitro* of Mg^{2+} and K^+ in rat uterus: ion pump activity. *Biochim. biophys. Acta* **291**, 260–268.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MOORE, R. D. (1973). Effect of insulin upon the sodium pump in frog skeletal muscle *J. Physiol.* **232**, 23–45.

NARAHARA, H. T. & ÖZAND, P. (1963). Studies of tissue permeability. IX. The effect of insulin on the penetration of 3-methylglucose-H^3 in frog muscle. *J. biol. Chem.* **238**, 40–49.

NIZET, A., JEFEBVRE, P. & CRABBÉ, J. (1971). Control by insulin of sodium, potassium and water excretion by the isolated dog kidney. *Pflügers Arch. ges. Physiol.* **323**, 11–20.

ÖZAND, P. & NARAHARA, H. T. (1964). Regulation of glycolysis in muscle. III. Influence of insulin, epinephrine, and contraction on phosphofructokinase activity in frog skeletal muscle. *J. biol. Chem.* **239**, 3146–3152.

POST, R. L., GEGYVARY, C. & KUME, S. (1972). Activation by adenosine triphosphatase in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. *J. biol. Chem.* **247**, 6530–6540.

ROGUS, E., PRICE, T. & ZIERLER, K. L. (1969). Sodium plus potassium-activated ouabain-inhibited adenosine triphosphatase from a fraction of rat skeletal muscle, and lack of insulin effect on it. *J. gen. Physiol.* **54**, 188–202.

SAUDEK, C. D., BOULTER, P. R., KNOPP, R. H. & ARKY, R. A. (1974). Sodium rentention accompanying insulin treatment of diabetes mellitus. *Diabetes* **23**, 240–246.

SKOU, J. C. (1962). Preparation from mammalian brain and kidney of enzyme system involved in active transport of Na^+ and K^+. *Biochim. biophys. Acta* **58**, 314.

SKOU, J. C., BUTLER, K. W. & HANSEN, O. (1971). The effect of magnesium, ATP, P_i, and sodium on the inhibition of the $(Na^+ + K^+)$-activated enzyme system by g-strophanthin. *Biochim. biophys. Acta* **241**, 443–461.

SWANSON, P. D., BRADFORD, H. F. & MCILWAIN, M. M. (1964). Stimulation and solubilization of the sodium ion-activated adenosine triphosphatase of cerebral microsomes by surface-active agents, especially polyoxyethylene ethers: actions of phospholipases and a neuraminidase. *Biochem. J.* **92**, 235–247.

TRIVEDI, B. & DANFORTH, W. H. (1966). Effect of pH on the kinetics of frog muscle phosphofructokinase. *J. biol. Chem.* **241**, 4110–4114.

WALAAS, O., WALAAS, E. & WICK, A. N. (1969). The stimulatory effect by insulin on incorporation of ^{32}P radioactive inorganic phosphate into intracellular inorganic phosphate, adenine nucleotides and guanine nucleotides of the intact isolated rat diaphragm. *Diabetologia* **5**, 79–87.

ZIERLER, K. L. (1959). Hyperpolarization of muscle by insulin in a glucose-free environment. *Am. J. Physiol.* **197**, 524–526.

ZIERLER, K. L. (1972). Insulin, ions and membrane potentials. In *Handbook of Physiology*, vol. I, section 7, pp. 347–368. Washington, D.C.: American Physiological Society.

ZIERLER, K. L. & RABINOWITZ, D. (1964). Effect of very small concentrations of insulin on forearm metabolism. Persistence of its action on potassium and free fatty acids without its effect on glucose. *J. clin. Invest.* **43**, 950–962.

ZIERLER, K. L., ROGUS, E. & HAZELWOOD, C. F. (1966). Effect of insulin on potassium flux and water and electrolyte content of muscles from normal and from hypophysectomized rats. *J. gen. Physiol.* **49**, 433–456.